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Functional, metal-based crosslinkers for α-helix induction in short peptides

Sarah J. Smith[#], **Kang Du**[#], **Robert J. Radford**, and **F. Akif Tezcan**[*]
Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Dr., La Jolla, California 92093-0356

[#] These authors contributed equally to this work.

Abstract

Many protein-protein interactions that play a central role in cellular processes involve α-helical domains. Consequently, there has been great interest in developing strategies for stabilizing short peptides in α-helical conformations toward the inhibition and interrogation of protein-protein interactions. Here, we show that tridentate Hybrid Coordination Motifs (HCMs), which consist of a natural (histidine, His) and an unnatural (8-hydroxyquinoline, Quin) metal binding functionality, can bind divalent metal ions with high affinity and thereby induce/stabilize an α-helical configuration in short peptide sequences. The Quin functionality is readily introduced onto peptide platforms both during or after solid-state peptide synthesis, demonstrating the preparative versatility of HCMs. A systematic study involving a series of HCM-bearing peptides has revealed the critical importance of the length of the linkage between the Quin moiety and the peptide backbone as well as the metal coordination geometry in determining the extent of α-helix induction. Through Zn^{II} coordination or modification with $Re^{I}(Quin)(CO)_3$, the HCM-bearing peptides can be rendered luminescent in the visible region, thus showing that HCMs can be exploited to simultaneously introduce structure and functionality into short peptides.

Introduction

Many natural protein-protein interactions (PPIs) are mediated by α-helical recognition and binding motifs on protein surfaces.[1] Because of the central involvement of PPIs in all cellular processes, considerable research activity has focused on designing non-natural,[2] protein-[3] or peptide-based platforms that structurally and chemically mimic α-helical protein surface motifs.[4] A drawback of using small peptidic platforms is that they are generally unstructured and susceptible to proteolytic cleavage.[5] To overcome this challenge, a number of strategies have been devised to stabilize them in α-helical conformations. These strategies include the incorporation of α–amino acids with restricted conformation space,[6] inclusion of salt-bridging residues in *i/i+4* positions,[7] crosslinking of side chains through covalent bonds[8] or metal-coordination,[9, 10, 11, 12] and utilization of hydrogen bond surrogates.[13] Each of these platforms has its own set of advantages concerning ease of preparation, the extent of protein modification and α-helix induction, stability, target recognition and *in vivo* uptake, but none of them simultaneously offer all of these advantages or are universally applicable. Moreover, in most instances, these helix induction motifs solely serve a structural purpose, with little functional value added to the peptide. Therefore, it would be desirable to have

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Correspondence to: F. Akif Tezcan.
[*]To whom correspondence should be addressed.

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access to alternative experimental platforms for helix induction that are easy to incorporate and modify, while simultaneously allowing the peptide to be functionalized and its secondary structure and other physicochemical properties to be easily modified.

Toward this goal, we present here short peptides that display Hybrid Coordination Motifs (HCMs), which are tridentate metal coordination modules consisting of a natural metal-coordinating residue such as histidine (His) at position *i* and an unnatural, bidentate chelating group such as 8-hydroxyquinoline (Quin) or 1,10-phenanthroline (Phen) covalently attached to the side chain at position *i*+7 (Figure 1). We first introduced HCMs as high-affinity coordination units on the surface of a folded, four-helix-bundle protein (cytochrome cb_{562}) to mediate protein self-assembly upon metal coordination.[14, 15] These studies revealed that the stability of the entire cyt cb_{562} fold increased by up to 4 kcal/mol upon binding of various divalent transition metal ions (Co^{II}, Ni^{II}, Cu^{II} and Zn^{II}), suggesting that HCMs might also be employed toward induction of α-helicity in unstructured peptides while allowing the incorporation of metal-based functionalities. Here, we have undertaken a systematic study in which we explored the chemical and physical properties of 10-residue-long peptides decorated with *i/i+7* His/Quin HCMs. We describe that the Quin moiety can be readily installed onto peptides either after solid-phase peptide synthesis (SPPS) via coupling to a cysteine (Cys) side chain or in the form of an unnatural amino acid during SPPS. We have found that the induction of α-helicity is critically dependent on the linker length between the Quin moiety and the peptide backbone, and that the extent of α-helicity can be controlled by the choice of the coordinating metal ion. The His/Quin HCMs can be rendered luminescent by through Zn^{II} binding, or alternatively, via coordination to a luminescent and substitution-inert $Re^{I}(Quin)(CO)_3$ moiety following SPPS. Finally, it is shown that His/Quin HCMs can induce considerable α-helicity in a peptide sequence that mimics the BH3 domain of the pro-apoptotic protein Bax. Thus, HCMs represent a versatile, easily implementable platform that allows simultaneous induction/modulation of α-helicity and the introduction of metal-based functionalities into peptide sequences.

Results and Discussion

Preparation of peptides containing His/Quin HCMs

General—The sequence and basic architecture of the HCM-bearing peptide platforms we have investigated are shown in Figure 1. All constructs are 10-residue, alanine (Ala)-rich peptides that were synthesized with a C-terminal amide and an acylated N-terminus via SPPS using standard Fmoc protecting group chemistry. Complete details on synthesis, peptide modification, purification and chemical analysis can be found in Supporting Information (SI). In general, the peptide sequences contained a His residue at position 2 (*i*) and a Quin-functionalized sidechain at position 9 (*i+7*), which would be two turns away in an α-helical configuration. The parent sequence also contained a lysine (Lys) at position 4 and a glutamic acid (Glu) at position 8 to form a potential salt bridge on a second face of the helix to further promote α-helicity and to increase the solubility of the peptide.

Routes for the incorporation of Quin functionality—The Quin functionality was introduced in two different ways: either during SPPS in the form of a pre-synthesized unnatural amino acid or after SPPS through covalent coupling to a Cys residue. For the former strategy, two Quin-bearing, Fmoc-protected amino acids (EQuin and EMeQuin, Figure 1b) were first prepared through the HATU-mediated coupling of 5-amino-8-hydroxyquinoline or 5-aminomethyl-8-hydroxyquinoline to Fmoc-protected L-Glu (see SI for synthetic details). EMeQuin presents the Quin functionality one extra methylene group removed from the peptide backbone compared to EQuin. The third Quin variant, CQuin, was obtained through the covalent coupling of 5-iodoacetamido-8-hydroxyquinoline (IA-

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Quin) to a Cys at position 9 following SPPS, paralleling our previous strategy to place a Quin moiety on the surface of the cytochrome *cb₅₆₂*.[14] CQuin, like EMeQuin, is separated from the peptide backbone by five single bonds although two of these bonds are slightly longer thioether linkages. It is important to note that the pre-SPPS preparative route of EQuin and EMeQuin potentially affords flexibility for creating multiply-functionalized HCM-bearing peptides, and eliminates the complications associated with postsynthetic labeling of Cys residues on whole-length peptides. It should further be mentioned that Quin-functionalized L-amino acids such as Sox have been previously reported by Imperiali and colleagues,[16, 17] who placed these functionalities within β-turn peptides for Zn^{II} sensing. The short side chains of Sox derivatives, which feature only a single methylene group between the Quin moiety and the α-carbon, are ideal for placement into the interior of a folded peptide, whereas EMeQuin, EQuin, and CQuin functionalities are, by design, long enough to extend over two turns on the surface of an α-helix.

Control peptides—In addition to H-EMeQuin, H-EQuin and H-CQuin (where H stands for the His component of the HCM in the 2 position), we prepared two further sets of peptides to investigate a) the importance of the His component of HCM and b) the necessity of the Lys(4)-Glu(8) salt bridge in the induction of α-helicity. For (a), we prepared the constructs A-CQuin and A-EMeQuin, which have His(2) replaced with a non-coordinating alanine (Ala) residue. For (b), we prepared H-CQuin-A and H-EMeQuin-A, which include an Ala in place of Glu(8).

Metal Binding Properties of His/Quin HCMs

We first studied the binding thermodynamics of Co^{II}, Ni^{II}, Cu^{II} and Zn^{II} to the three HCM-bearing peptides, H-EMeQuin, H-CQuin and H-EQuin, by monitoring the 20-nm red shift of the π-π* absorption band of Quin (λ_{max} = 244 nm) upon metal coordination (Figure S7). As we previously observed with HCMs installed on the cytochrome cb_{562} surface,[14, 15] the binding affinities of the HCM-bearing peptides were too high to be measured by direct titrations. Therefore, EGTA (ethylene glycol tetraacetic acid) or ADA (*N*-(2-acetamido)iminodiacetic acid) was included in the titration as a competing ligand. ADA was used in cases where metal-peptide binding was not strong enough to compete with EGTA, specifically in the cases of Co^{II} and Zn^{II}. Under our experimental conditions, which included 10-20 μM peptide, 30-60 μM EGTA or ADA, and 0-60 μM M^{II}, all binding curves were best described using a model that simultaneously took into account a 1:1 peptide:metal binding equilibrium (P:M) and a 2:1 metal-mediated peptide dimerization event (P:M:P) (Figure S8 and Scheme S6). In all cases studied, the dissociation constant (K_d) for metal-mediated peptide dimers was determined to be in the low μM range.

The dissociation constants of the metal-HCM complexes (P:M) range from nanomolar for Co^{II} and Zn^{II} to femtomolar for Cu^{II} (Table 1). These K_d's are two to three orders of magnitude lower than those for the free Quin ligand (Table 1),[18] indicating the formation of the intended tridentate coordination mode that we previously observed in the crystal structure of a cytochrome cb_{562} variant with a surface H-CQuin motif.[14, 15] Notably, the metal binding affinities of the HCM-peptides are three to seven orders of magnitude higher than those observed for similar peptides bearing $i/i+4$ bis-His chelation motifs[9, 19] highlighting the dramatic effect of increased denticity on metal affinity. In general, the metal binding affinities of H-EMeQuin, H-CQuin and H-EQuin roughly follow the Irving-Williams series ($Co^{II}<Ni^{II}<<Cu^{II}>>Zn^{II}$). The affinity of H-EMeQuin for each metal ion is consistently lower (by one to two orders of magnitude) than those of H-CQuin and H-EQuin, which we attribute to specific interactions between the HCMs and the peptide surface. Quantum mechanics/molecular mechanics (QM/MM) calculations are currently

underway to gain structural/energetic insights into H-EMeQuin, H-CQuin and H-EQuin and their metal complexes.

Metal-Induced α-Helicity in HCM-containing Peptides

Metal-induced changes in the peptide secondary structure were monitored by circular dichroism (CD) spectroscopy (Figure 2, see Figures S9 and S10 for spectra including all metals), initially at 4 °C. In these experiments, a low peptide concentration (10 μM) and a 3-fold excess of Co^{II}, Ni^{II}, Cu^{II}, or Zn^{II} ensured that metal-induced peptide dimerization was not a concern and that each peptide was fully bound to a metal ion. In the absence of metal binding, H-CQuin, H-EMeQuin, and H-EQuin all displayed a CD signature reflective of a random coil conformation with a minimum at 195 nm. Upon addition of metal ions, the spectrum for H-EQuin showed little to no change from the random coil conformation. In contrast, H-CQuin and H-EMeQuin, whose HCMs are one bond longer than H-EQuin, displayed a significant induction of α-helicity as indicated by the emergence of two characteristic minima at ~206 and 222 nm.[20] Of all metal ions tested, Cu^{II} induced the greatest extent of helicity in H-CQuin and H-EMeQuin, followed by Zn^{II}, Ni^{II}, and Co^{II} in descending order (Figure 2, Figures S9 and S10). In all cases, stoichiometric metal coordination to the Quin functionality was confirmed by full red-shift of the Quin π-π* absorption band from 244 nm to 264 nm.

Upon replacement of the His component of the HCMs with an Ala to obtain A-CQuin and A-EMeQuin, metal-induced changes in the CD spectra were abolished (Figure 2, Figures S9 and S10). This finding confirmed that metal binding by the HCMs and the resulting cross-linking of the two-helix turn portion were responsible for α-helix formation. The extent of α-helicities of metal-bound H-CQuin and H-EMeQuin based on mean residue ellipticity at 222 nm are listed in Table S1, although such estimates have been reported to be inaccurate for short helices.[21] Therefore, we also obtained the CD spectra of H-CQuin and H-EMeQuin in the presence of 60% trifluoroethanol (TFE), a potent helix inducer.[22] Assuming that TFE produces a fully helical conformation, the Cu^{II}-complexes of H-CQuin and H-EMeQuin possess ~70% and ~100% helicity, respectively, at 4 °C, based on relative CD intensities at 222 nm. At 25 °C, the Cu^{II} complexes still retain significant secondary structure (H-CQuin, 54% helical; H-EMeQuin, 88% helical).

In order to determine whether the Lys(4)-Glu(8) salt bridge is necessary for α-helix formation and the extent of its contribution to the peptide secondary structure, we examined the constructs H-EMeQuin-A and H-CQuin-A. The CD spectra of H-EMeQuin-A and H-CQuin-A showed that metal-induced helix formation was not affected by the elimination of the Lys(4)-Glu(8) salt bridge (Figure 2, Figures S9 and S10): the helicities of both peptides ranged from ~80% in the presence of Cu^{II} to ~40% in the presence of Co^{II} (Table S1). In fact, Zn^{II} binding induced more helicity for both peptides relative to the parent constructs containing salt bridges. Taken together, our findings clearly indicate that metal binding by the *i/i+7* HCMs alone can induce considerable α-helicity in short helices. Importantly, HCMs occupy only one of the three available faces of an α-helix, meaning that residues facing the other two faces can be used for the incorporation of other functionalities and for target recognition.

Functionalization of HCMs

Having shown that H-CQuin and H-EMeQuin motifs tightly bind divalent transition metal ions and induce an α-helical peptide conformation in doing so, we next investigated whether they can be simultaneously exploited for incorporating metal-based functionalities. In a rare previous example where this has been achieved, the Ball group has employed dirhodium complexes to induce helicity in short, unstructured peptides upon coordination to *i/i*+3 and *i/*

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i+4 Glu or Asp pairs;[11] the catalytic activity of these dirhodium-peptide conjugates were reported in separate studies.[23] In our case, we initially set out to take advantage of the Quin functionality, whose derivatives have long been employed in various analytical applications owing to their intense fluorescence that is activated in selective response to metal ions,[24] in particular to Zn^{II}.[25, 26] Indeed, H-CQuin displayed a ~5-fold increase in fluorescence intensity at 540 nm upon Zn^{II} coordination, but not in the presence of Co^{II}, Ni^{II}, or Cu^{II} (Figure 3a). As calculated with reference to a quinine sulfate standard, the quantum yield (φ_{540nm}) of Zn^{II}-H-CQuin is 0.27%, which is comparable to that of the Zn^{II} complex of free 8-hydroxy-2-methylquinoline (0.4%).[26] The amine group directly attached to the quinoline ring in H-CQuin likely quenches fluorescence by photoinduced electron transfer and is therefore partially responsible for the low quantum yield.[27] In accordance with this proposal, H-EMeQuin, in which the amine group is one methylene unit removed from the aromatic ring, showed a ~20-fold fluorescence enhancement upon Zn^{II} binding over background (Figure 3b), with an improved φ_{540nm} of 0.64%. Further, regardless of His coordination, peptides tested containing a methylene spacer (A-EMeQuin, H-EMeQuin), consistently showed higher fluorescent intensity than those peptides with the amine group directly attached to the quinoline ring (H-CQuin, A-CQuin, H-EQuin) (Figure 3b).

Aside from coordinating aquated transition metal ions, the tripodal coordination motif of HCMs should also stably accommodate metal complexes that possess the appropriate geometry and could be used to endow the peptide scaffolds with additional functionalities. In particular, complexes of second- and third-row transition metals (Re^{I}, Ru^{II}, Os^{II}, Ir^{III} and Rh^{III}) display rich photophysical properties such as long lifetimes and high photostabilities[28] that have been exploited in biological imaging,[29, 30] sometimes in the form of metallopeptide conjugates.[31, 32] The direct integration of such metal complexes into a peptide crosslinking moiety would obviate the need for additional functional groups (i.e., fluorophores), which have been shown to influence the cellular localization of peptides.[33] Additionally, incorporation of these capped, substitution-inert metal complexes would eliminate the possibility of undesired metal-mediated peptide dimerization and yield kinetically stable conjugates that could persist in the intracellular environment, which is deprived of free metal ions.

To demonstrate the ability of HCMs to anchor functional metal complexes, we synthesized the luminescent $Re^{I}(IA\text{-}Quin)(CO)_3X$ compound,[34] which could be directly coupled to Cys(9) following SPPS with >90% yield (Figure 4a). The product, purified by HPLC, had a mass of 1453.80 amu (theoretical mass = 1490.10 amu), which is consistent with the loss of the chloride ligand either during the MS experiment or earlier during purification through substitution by a solvento species. Because Re^{I} is substitution-inert, we first heated the resulting Re-peptide conjugate to 65 °C in order to promote His(2)-Re coordination, then cooled it down to room temperature, while monitoring the α-helicity of the peptide by CD spectroscopy at 222 nm (Figure 4 a). Upon the completion of the heating-cooling cycle, the α-helicity of the sample significantly increased, suggesting that the Re^{I}-HCM was successfully formed (Figure 4b, 4c). The product displayed a visible band at ~410 nm (Figure S13), whose excitation produced two luminescence bands with maxima at 550 nm and 680 nm (Figure 4d; see Figure S14 for changes in the luminescence spectrum upon heating). These spectral features accord very well with those of the model complex, $Re^{I}(Quin)(CO)_3$(pyridine), which was reported to have an absorption band centered at 420-430 nm, a fluorescence band at ~530 nm and a phosphorescence band 670-690 nm.[35] The IR-spectrum of the Re-HCM peptide was also very similar to that of the model complex, with three maxima at 1888, 1903, and 2016 cm^{-1} corresponding to the three carbonyl groups in a *facial* arrangement (Figure 4e). These results confirm the intended coordination geometry of the Re^{I}-HCM complex and establish that His-Quin HCMs can be used to harbor functional metal complexes on an α-helical peptide platform.

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HCM-mediated helicity in non-Ala-rich peptide sequences

Although the HCM-peptide sequences presented thus far all a display random coil signatures in the absence of metal coordination, it is well documented that Ala-rich sequences have high propensity for α-helix formation.[36] Therefore, we sought to determine whether our HCM strategy could be employed to induce helicity also in non-Ala-rich peptide sequences that may have downstream applications. To this end, we synthesized an HCM-bearing peptide sequence (Bax-HCM) based on the helical BH3 (Bcl-2 homology domain 3) motif of the pro-apoptotic protein Bax. Because BH3 domains play a central role in the interactions between Bcl-2 family proteins involved in the regulation of cell death, structural mimics of BH3 domains have received considerable attention as potential cancer therapeutics[37] and therefore constituted an interesting proof-of-principle target for our strategy. Bax-HCM, a 26-residue sequence, contained the entire helical BH3 domain (residues 55-80, Figure 5) of Bax which is necessary and sufficient for interaction with Bcl-2.[38] The only two variations from the wildtype protein sequence were Ser^{72}→His and Met^{79}→EMeQuin for the construction of the *i/i*+7 HCM motif on the face of the BH3 helix that does not interact with the target protein. CD experiments indicated that Bax-HCM (10 μM in concentration) displayed little–if any–helicity in the absence of metals (Figure 5). Upon addition of metal ions, we observed a clear emergence of an α-helical signature, the effect being largest with Cu^{II}, followed by Zn^{II}, Co^{II} and Ni^{II} (Figures 5, S9 and S10), roughly following the same trend observed with the 10-mer sequences. Notably, in contrast to these short Ala-rich peptides, Bax-HCM displayed no appreciable difference in helicity between 4 °C and 25 °C (Figure 5), which may be attributable to its longer length.

Conclusions

In summary, we have presented here the synthesis and characterization of various Quin-based HCM-bearing peptides and their metal-dependent structural and photophysical properties. There is rich and extensive literature on metal-induced α-helicity in peptides,[9, 10, 39] metal-peptide conjugates,[32, 40] and functional metallopeptides.[16, 41] Our strategy of building HCMs on peptide sequences borrows from all of these somewhat non-overlapping efforts to provide a unique combination of advantages for creating functional, α-helical structures. HCM-bearing peptides are readily prepared via multiple routes with high yield in a few synthetic steps from commercially available components. HCMs are modular in the sense that they can be interchangeably (i.e., reversibly) complexed with various divalent transition metal ions or metal complexes whose different coordination properties can be utilized to control the α-helicity of the peptide scaffold. Though not explored here, HCMs could also allow the control of peptide conformation through changes in solution pH or redox potential (as both of these factors can modulate inner-sphere metal coordination) so as to construct stimuli-responsive peptide platforms. Based on the two sets of peptides we studied here (the 10mer, Ala-rich sequences and the 26mer Bax BH3 domain), HCMs appear to be generally applicable for helix induction, with the added advantage that the “helix staple” can now carry an intrinsic and useful functionality such as luminescence.

On a final note, given the tight regulation and scarcity of uncomplexed transition metal ions in the intracellular environment, the use of α-helical HCM peptides containing labile metal ions (e.g., Co^{II}, Ni^{II}, Cu^{II} and Zn^{II}) may be more useful for *in vitro* experiments or for targeting extracellular proteins such as cell surface receptors. Nevertheless, as we have demonstrated with a Re^{I}-carbonyl compound, HCM peptides are readily modified with substitution-inert metal complexes, which should not only yield stable α-helical peptides, but also may lend their intrinsic physical properties and chemical reactivities toward diagnostic and therapeutic *in vivo* applications.[29]

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Supplementary Material

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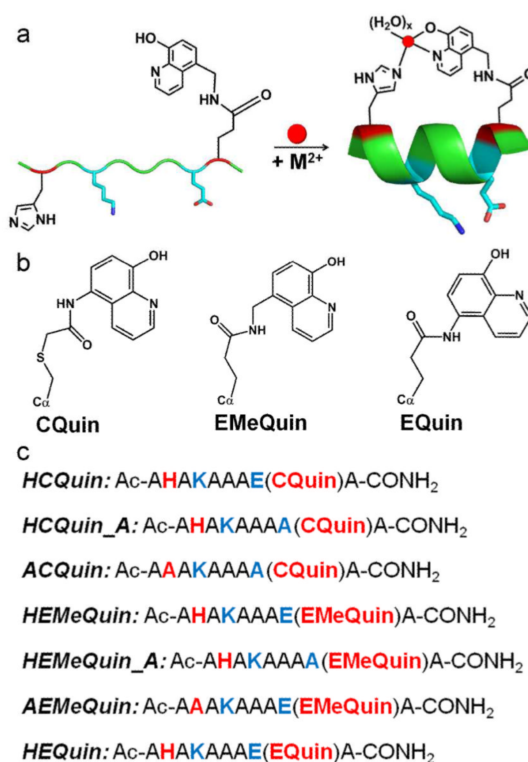

Figure 1.

(a) Proposed scheme for α-helix induction through tridentate metal coordination by an HCM. The remaining coordination sites on the metal are likely filled by aquo ligands. **(b)** Chemical structures of various Quin functionalities. **(c)** Sequences of peptide constructs prepared in this study. The coloring scheme corresponds to that in (a).

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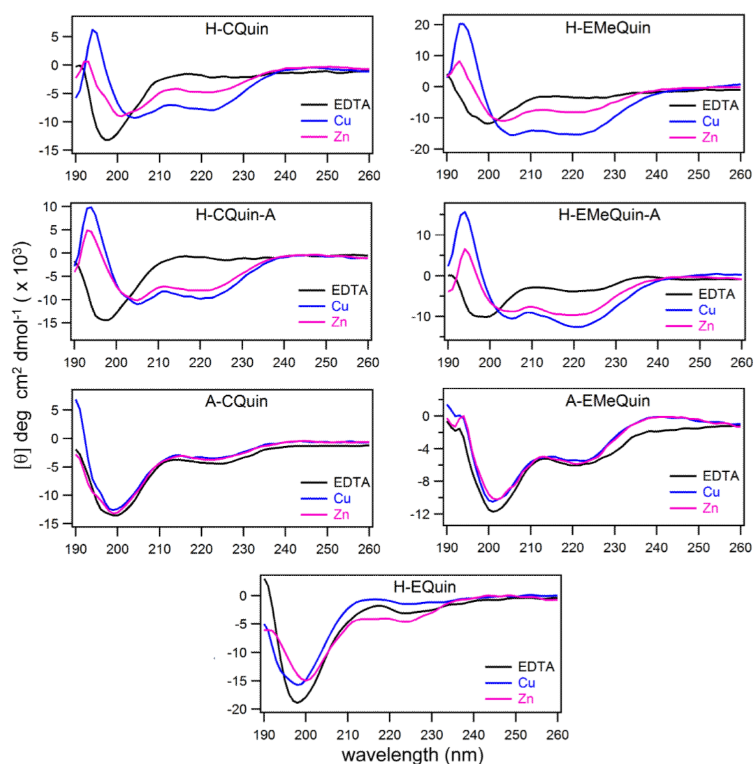

Figure 2.

Changes in the circular dichroism spectra of HCM-bearing peptides upon binding Cu^{II} and Zn^{II}. Spectra were acquired at 4 °C with 10 μM peptide and 30 μM EDTA or metal in 5 mM sodium borate buffer at pH 7.5. Corresponding spectra for Co^{II} and Ni^{II} are shown in Figure S9 and S10.

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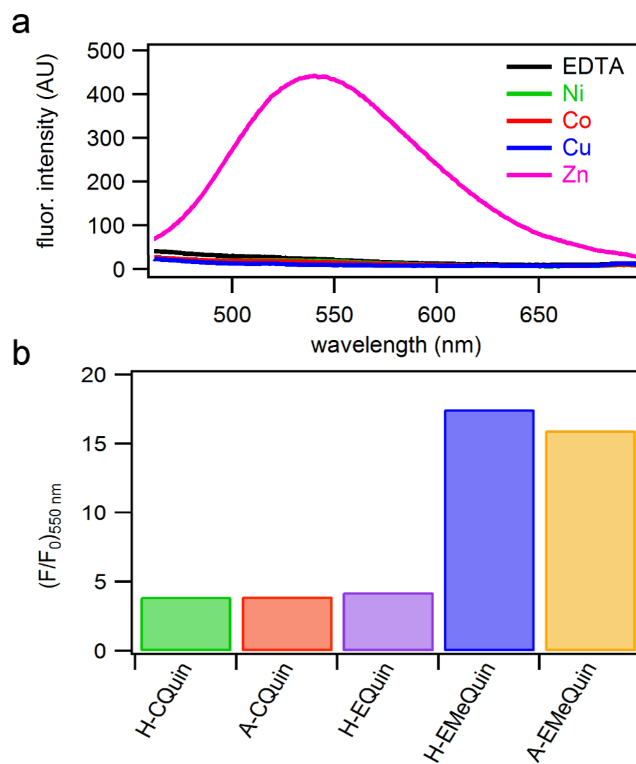

Figure 3.
(a) H-EMeQuin fluorescence upon metal addition (λ_{exc} = 385 nm). **(b)** Normalized fluorescence intensity (F/F_0) of each peptide when bound to Zn^{II} as compared to the metal-free peptide.

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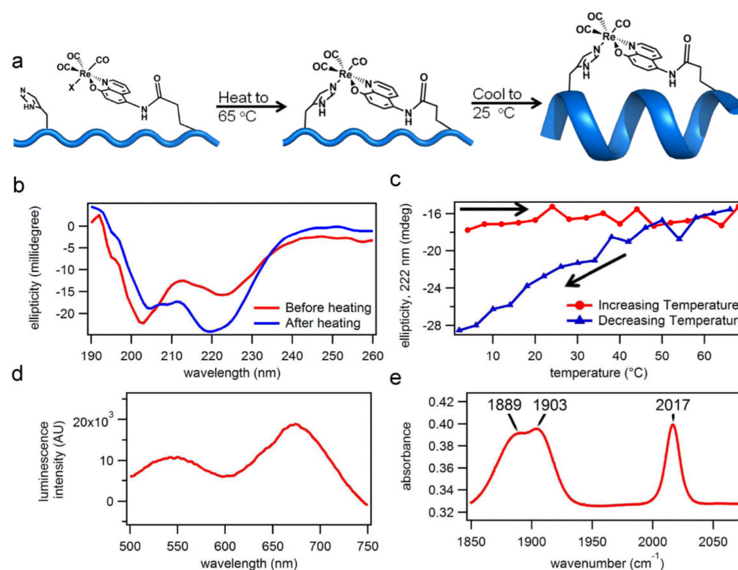

Figure 4.

(a) Proposed scheme for the formation of the $Re(Quin)(CO)_3(His)$ HCM upon heating and the subsequent formation of the α-helix upon cooling. **(b)** Observed changes in the CD spectrum upon the heating and subsequent cooling of the Re-HCM peptide. **(c)** Changes in the CD signal (222 nm) during heating and cooling. **(d)** The emission spectrum of the Re-HCM peptide obtained after the heating/cooling cycle (λ_{exc} = 410 nm). **(e)** The IR spectrum of the Re-HCM peptide. The peptide sample was first lyophilized and then combined with KBr to make a pellet.

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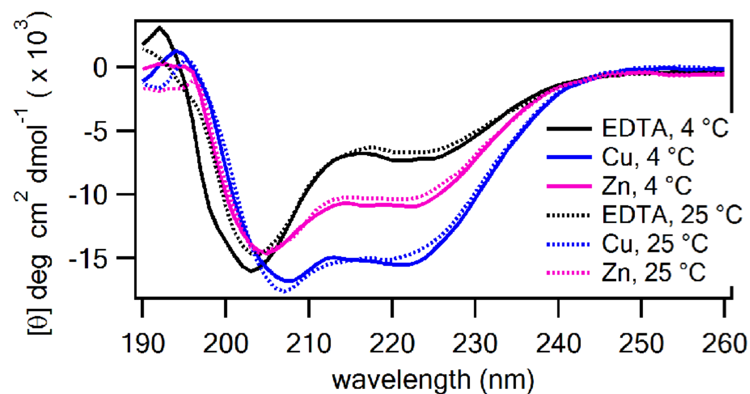

Figure 5.

Sequences of wild-type and HCM-modified Bax BH3 domain peptides (top). Far-UV CD spectra of the Bax-HCM in the presence and absence of Cu^{II} (blue) and Zn^{II} (magenta) acquired at 4 °C (solid lines) or 25 °C (dashed lines). The samples contained 10 μM peptide and 30 μM EDTA or metal ion in a 5 mM sodium borate buffer solution at pH 7.5.

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Table 1

Dissociation constants for various peptide-metal complexes. Number in parentheses correspond to standard deviation in the last reported significant figure.

	Dissociation Constants (M)			
	H-CQuin	**H-EMeQuin**	**H-EQuin**	**Quin[18]**
Co^{II}	$2\ (1) \times 10^{-9}$	$1.2(5) \times 10^{-8}$	$2\ (1) \times 10^{-9}$	6.5×10^{-7}
Ni^{II}	$2\ (1) \times 10^{-11}$	$1.1(1) \times 10^{-9}$	$2(1) \times 10^{-10}$	1.6×10^{-7}
Cu^{II}	$5.2(4) \times 10^{-14}$	$1.4(8) \times 10^{-13}$	$6.3(1) \times 10^{-14}$	2.3×10^{-10}
Zn^{II}	$6.2(1) \times 10^{-9}$	$6.8(3) \times 10^{-9}$	$3\ (1) \times 10^{-9}$	8.7×10^{-7}