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Inducing α -Helicity in Peptides by Silver Coordination to Cysteine

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Short peptide sequences consisting of two cysteine residues separated by three other amino acids display complete change from random coil to α -helical secondary structure in response to addition of Ag⁺ ions. The folded CXXXC/Ag⁺ complex involves formation of multinuclear Ag⁺ species and is stable in a wide pH range from below 3 to above 8. The complex is stable through reversed-phase HPLC separation as well as towards a physiological level of chloride ions, based on far-UV circular dichroism spectroscopy. In electrospray MS under acidic conditions a peptide dimer with four Ag⁺ ions bound was

Introduction

Coordination of metal ions to peptides or proteins often leads to stabilization or change of their spatial structure. Some proteins have a specific metal binding motif which upon coordination may activate it for a specific task. The transcriptional metalloregulatory protein CueR, for example, responds to elevated cellular levels of the monovalent group 11 transition metals (Cu⁺, Ag⁺, and Au⁺) by binding to the ions via its metalbinding loop and activating transcription of the *cue* operon which regulates metal ion homeostasis.^[1] On the other hand, coordination of metal ions may also severely disrupt the structure and function of proteins, leading to toxic effects.^[2] Non-coordinated short linear peptides seldom possess welldefined secondary structure.

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observed, and modelling based on potentiometric experiments supported this to be the dominating complex at neutral pH together with a peptide dimer with 3 Ag⁺ and one proton at lower pH. The complex was demonstrated to work as a *N*-terminal nucleation site for inducing α -helicity into longer peptides. This type of silver-mediated peptide assembly and folding may be of more general use for stabilizing not only peptide folding but also for controlling oligomerization even under acidic conditions.

Macrocyclization of such peptides reduces their conformational flexibility, which has led to enhanced biological potency and selectivity, as well as enhanced stability towards degradation. For example, pentapeptide motifs have been shown to adopt α -helical structures in response to various types of covalent macrocyclization between residue 1 and 5 (*i*, *i*+4).^[3] Rigidification via electrostatic interactions^[4] or metal coordination between side-chains are other ways of stabilizing secondary structure in peptides. Almost all examples of latter strategy utilize two metal coordinating residues in an *i*, *i*+4 constellation, corresponding to one turn of an α -helix (Scheme 1). Several reports describe short peptides with histidine (His), cysteine (Cys), and/or methionine (Met) residues coordinated to divalent metal ions such as Cd²⁺, Zn²⁺, Cu²⁺, Ni²⁺, Co²⁺, Pd²⁺ or Ru^{2+,[S]}

Short α -helical peptides are of interest as some are capable of binding to and disrupting cell membranes and thus have



Scheme 1. Previous and present work. A: Coordinating divalent metals to short peptides. B: Structures of the peptides (1–4) applied for initial screening of Ag⁺ coordination as well as the disulfide peptide 2 (oxidized form of 1).

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potential in the development of new antimicrobial agents.^[6] Inserting two Zn^{2+} -coordinating His residues in an *i*, *i*+4 constellation in mastoparan X for example, stabilized its α -helical structure and led to increased lytic activity.^[7] There are several examples of longer peptides from biological systems where a helix-loop-helix fold is maintained by two disulfide bridges in sequences having two CXXXC motifs, e.g. peptidebased K-channel toxins.^[8] Metal-binding bis(cysteinyl) sequences are also common in Nature, whereas designed peptide sequences incorporating two free Cys residues are scarce. An example of metal-induced structure formation is the sequence Ac-SCHGDQGSDCSI-NH₂ which forms a loop structure in response to Hg^{2+.[9]} Cys residues have been incorporated in TRI peptides in CXXC and CXXXC metal-binding motifs, binding Cd²⁺ and Hg²⁺. In this case, however, metal coordination stabilized already existing helical complexes or led to the formation of new aggregates.^[10] Várnagy et al. have studied a range of short CX_nC peptides and their metal binding abilities towards several divalent metal ions, but not to singly charged noble metals.^[11] Ag⁺-mediated switching of peptide secondary structure is not well-described in the literature, although it has been shown that the intrinsically disordered protein SilE adopts a highly ordered α -helical structure in response to Ag⁺ ions by coordination to its His and Met residues.^[12] Also, a Cyscontaining zinc finger motif has recently been shown to adopt a partially refolded α -helical motif around multinuclear Ag_nS_n clusters in response to the replacement of Zn²⁺ by several equivalents of $Ag^{+,[13]}$. In contrast, formation of Ag_nS_n clusters from the zinc hook domain of the Rad50 protein have been shown to disrupt its structure.^[14] Examples of multinuclear Cu⁺ peptide complexes that may be structurally similar to corresponding Ag⁺ systems have also been reported.^[15] Silver in the form of Ag⁺ or nanoparticles is well described as antibacterial agent and has previously been broadly applied for medicinal purposes.^[16] The molecular mechanism for the biological effect of silver is far from fully elucidated. In general, the effects of Ag⁺ are attributed to disruption caused by the replacement of Zn²⁺ and other essential protein-bound metal ions. Based on the case of CueR, in which C112 and C120 of the metal-binding loop facilitate the binding of Cu⁺, Ag⁺, and Au⁺, coordination of silver to proteins and peptides could also be initiating pathways. With the aim of understanding how silver coordination to cysteine residues may stabilize peptide and protein structure we have explored a coordination-induced secondary structure motif in CX_aC peptides where folding is achieved by metal binding to the two cysteines.

Results and Discussion

Initially, the peptide Ac–RCAAAC–NH₂ (1) was explored for metal coordination as this sequence previously was used for studying the structure of covalent macrocycles.^[17] A series of other small peptides (**3–5**) also containing two cysteine residues but separated by varying numbers of consecutive Ala residues were investigated as well (Scheme 1).

The apopeptides 1, 3 and 5 (Figure 1) all have CD spectra with minima at just below 200 nm, equivalent to peptides with random coil structures. It was found that addition of excess Ag⁺ to the peptide 1, which have the two cysteine residues in the *i*,*i*+4 positions, led to a major change in solution structure equivalent to a change from an unstructured to a highly α -helical peptide (Figure 1B). The disulfide-bridged macrocycle 2 (See SI Figure S62) as well as the remaining sequences (3, 4, and 5), where cysteine residues occupy the *i*, *i*+2, *i*, *i*+3, or *i*, *i*+5 positions, respectively, displayed less or no distinct structural change upon addition of Ag⁺ (Figure 1A, 1C and 1D). However, it was noted that the CD spectra of peptide 4 displayed some features reminiscent of beta-turn structure with a negative peak at 230 nm and positive peak at 210 nm.^[18]

The CD intensity at 222 nm has long been applied as a gauge for alpha-helical content in lack of more comprehensive models for short peptides. Hence, it is possible to estimate the degree of folding that takes place for 1 with addition of Ag⁺ (Figure 1B). Using the original and still widely applied formula published by Chen et al in 1974^[19] (see SI for further details) yields a fraction helicity of 71% (f_{H}\!=\!0.71) for 1 (pH 7, 2 equiv. Ag⁺). Several improvements have been proposed to this formula including a temperature-correction term among other things.^[20] Taking this into account the value $f_{H} = 0.72$ is obtained for 1 (pH 7, 2 equiv. Ag⁺) at 25 °C. Analysis of CD data may also give information on interaction between helices, i.e. coiled-coil formation.^[21] According to literature, UV CD signals may be interpreted via the ratio between the two minima in the spectrum of an alpha-helical fold by taking the ellipticity at 222 nm relative to 208 nm, such that $\theta_{222 nm}/\theta_{208 nm} \ge 1$ for coiled coils and $\theta_{222\,nm}\!/\!\theta_{208\,nm}\!\le\!0.86$ for isolated helices. For 1 (pH 7, 2 equiv. Ag⁺) a ratio of 1.3 were calculated indicating coiled-coil formation. However, in case of a short peptide binding two equivalents of silver ions there is a likelihood of far UV spectral contributions from e.g. charge-transfer or other absorbance bands involving Ag⁺ which are not related to pure peptide backbone electronic transitions.

The structural effects of the concentration ratio between 1 and ${\rm Ag}^+$ were explored by CD, HPLC, LC-ESI-MS, and NMR







experiments. CD analysis at neutral pH revealed that a sudden change starts occurring above a 1.5:1 Ag⁺/1 ratio with a stable high degree of α -helicity from a 2:1 ratio and upwards (Figure 2). Reversed-phase HPLC analysis demonstrated that addition of 0.5 to 1.5 equivalents of Ag⁺ to 1 gave rise to a new very broad signal with longer retention time than that of 1 (Figure 2) as well as **2** (SI Figure S10).

Further addition of Ag^+ led to a reduction in the retention time and a marked sharpening of the signal. At a $Ag^+/1$ ratio of



Figure 2. Top: Titration of 1 with Ag⁺ monitored by CD in 8 mM phosphate buffer, pH 7.2 shown as mean residue ellipticity at 222 nm and as far-UV CD spectra. Bottom: Titration of 1 with Ag⁺ monitored by RP-HPLC. The y-axis tick marks indicate values from 0 to 1 for all chromatograms.

2:1, the new signal achieved its maximal intensity. Increased retention time has previously been observed to correlate with increased α -helicity,^[22] which is probably due to shielding of the backbone. However, the initial species with the longest retention time seen up to a $Aq^+/1$ ratio of 1.5:1 are likely due to oligomer formation via Ag⁺ coordination. The HPLC titration also confirms that no oxidation to form a disulfide bond takes place in samples with Ag⁺ as **2** has a lower retention time than 1. UPLC-ESI-MS analysis showed a doubly charged dimer of 1 with four Ag⁺ ions bound, $[C_{46}H_{82}N_{20}O_{14}S_4Ag_4]^{2+}$ observed at m/z = 849 (SI Figures S71–73) which we attribute to the intense signal seen in HPLC when 2 equiv. Ag⁺ are added. Silver compounds can form complex structures, with the possibility of argentophilic interactions between silver ions^[23] and these may also be polymeric in systems with multiple thiolates.^[24] In zinc finger domains with four cysteine residues, a single-chain complex with four silver ions bound has been characterized. The sequence wraps around the silver cluster in an antiparallel fashion with one half of the backbone adopting α -helical folding.^[13] This could be in a structural analogy to two CAAACcontaining peptides forming a dimer in order to establish a similar S₄-Ag₄ coordination pattern as indicated by the ESI-MS data on 1. (See SI Figures S51-S54). Interestingly, the dimer with four Ag⁺ ions was observed at all Ag⁺ stoichiometries (0.5-2.5 equiv.) under acidic conditions (Figure S73). The fact that it was observed even under the acidic conditions of reversed-phase LC indicates high stability. It also became apparent that compared to other metal ions with high affinity for thiolate binding only Ag⁺ exerted this effect on peptide 1 as addition of Cd²⁺ or Hg²⁺ gave rise to random coil or unusual signals, respectively (SI Figure S59). LC-ESI-MS analysis also revealed that 1 formed a 1:1 adduct with Hg²⁺, whereas no adduct of Cd²⁺ was observed under LC conditions (SI Figures S55–S58). As seen by NMR spectroscopy the addition of Ag⁺ to 1 caused general broadening of peaks and the disappearance of the Cys H^{β} signals at 2.97–2.70 ppm in ¹H spectra (SI Figure S70), consistent with recent studies of Ag⁺ bound to glutathione.^[25] Two new broad signals at 4.18 and 2.10 ppm appeared with no further change above a $Ag^+/1$ ratio of 2:1.

All these results pointed to that the α -helical structure complex is a peptide dimer with four Ag^+ ions bound $(Ag^+/1)$ ratio of 4:2). However, it was found that at acidic pH a lower ratio of Ag⁺ is required to initialize the folding according to CD spectroscopy (see SI Figure 60A-61C). For more detailed information, a pH-potentiometric analysis was performed on 1 in the presence of varying equivalents of Ag⁺ (See Figure 3 and SI for data and discussion). These data confirm that the peptide binds Ag⁺ already from low pH as deprotonation of a significant fraction of the peptide thiols is observed even at 0.5 equiv. of Ag⁺ per peptide ligand. The system is complex and a possible model of the speciationindicates that various multinuclear Ag⁺ complexes are formed already at substoichiometric metal ion addition (SI Figure S75). It is proposed that the Ag⁺-binding pattern in dimeric, multinuclear species, detected also by ESI-MS promotes the switching of the secondary structure of 1 into an α -helix dominated conformation. In the Research Article doi.org/10.1002/chem.202304064



Figure 3. Analysis of pH-potentiometric data. Top: Titration curves of ~1 mM 1 in HClO₄ with 0, 0.5, 1.0, 1.5 and 2.0 equiv. of Ag⁺, depicted as consumed base equivalents per ligand (n_{OH-}/n_1) with excess HClO₄ titrated at negative values. Symbols and continuous lines represent the measured and fitted points, respectively. Bottom: Summarized ligand fraction of the species $[Ag_3HL_2]^{2+}$ and $[Ag_4L_2]^{2+}$, proposed to contribute to the observed α -helicity in the Ag⁺/1 system at pH = 7.0 (red curve) and at pH = 2.9 (blue curve), plotted as a function of the $c_{Ag+}/c_{peptide}$ ratio. The filled circles show the normalized molar ellipticities at 222 nm (right axis) recorded at pH = 7.0 (red) and 2.9 (blue).

analysis the fully protonated peptide is denoted $[H_2L]^+$ and $[L]^$ is thus the case when both Cys thiols are deprotonated. According to a plausible speciation model (Figure 3 and Figure S75) two types of dimeric species are formed, $[Ag_3HL_2]^{2+}$ and $[Ag_4L_2]^{2+}$. A structural interpretation could here be that a fourth Ag^+ ion replaces a bridging proton in the $[Ag_3HL_2]^{2+} \alpha$ helical complex to yield a more stable $[Ag_4L_2]^{2+}$ complex with the same overall peptide dimer structure. The distribution of the two complexes show an interesting pH dependence with a maximum of their summarized fraction at 2.0 equiv. and 1.5 equiv. of Ag^+ per ligand at pH ~7 and 3, respectively (Figure 3). This correlates well with the CD-intensities observed as a function of the $Ag^+/1$ concentration ratio under the same conditions.

In order to further assess the structure and strength of the Ag⁺/1 4:2 complex, isolation by liquid chromatography and lyophilization was undertaken. The isolated powder displayed unchanged α -helical structure once redissolved, though, the exact geometric structure of the complex was not elucidated as crystallization attempts were unsuccessful. However, a large

number of silver-thiolate clusters that have been characterized include compounds containing an octagonal Ag₄S₄ unit.^[26] When generating the complex at physiological mimicking conditions (PBS buffer, pH 7.4), a significant α -helical contribution to the CD signal was still observed, despite the strong competition from Cl⁻ for Ag⁺ binding (Figure 4A). CD measurements performed of 1 in phosphate buffer at pH 3.0 showed that clear α -helical folding was maintained with 2.0 equiv. Ag⁺ (Figure 4A, green curve). The impact of the cationic amino acid residue in the sequence was investigated by substituting the single arginine (Arg) residue with a lysine (Lys) residue and changing its position. For this purpose, the three peptides Ac-KCAAAC-NH₂ (6), Ac-ACARAC-NH₂ (7), and Ac-CAAACR-NH₂ (8) were synthesized. The CD spectra of these peptides (6-8) were similar to that of 1 (Figure 4) with the complex of **8** being the most α -helical. α -Helices are known to possess a dipole moment by virtue of the combined backbone



Figure 4. (A) CD spectra of 1 prior to (black) and after addition of 2 equiv. Ag⁺ in 8 mM phosphate buffer pH 7.2 (red), pH 3.0 (green), or PBS buffer pH 7.4 (blue dot). *The PBS curve was cut at ~200 nm due to UV absorption by the buffer at lower wavelengths. (B–F) CD spectra of 6, 7, 8, 9, and 10 with and without 2 equiv. Ag⁺, all in phosphate buffer pH 7.2.

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European Chemical Societies Publishing hydrogen bonds,^[27] with a C-terminal negative charge and an *N*-terminal positive charge. In **8**, with the positively charged Arg residue positioned C-terminally, induction of α -helicity is therefore expected to be more favorable due to charge dispersion.^[28]

The complex of Ag⁺ and the CAAAC motif was investigated for its ability to propagate α -helicity in longer peptides. For this purpose, the peptides Ac–[ARA]₃CAAAC–NH₂ (9) and Ac–CAAAC[ARA]₃–NH₂ (10) were synthesized and purified. These were based on model peptides used by Fairlie and coworkers.^[29] Addition of 2.0 equiv. Ag⁺ to peptide 9, with the CAAAC motif positioned *C*-terminally, resulted in only a partial α -helicity indicating a weak propagation. Peptide 10, on the other hand displayed a very strong α -helical signal (Figure 4F) under the same conditions, albeit the apopeptide did not appear completely random coil judged by the signal at 222 nm. This points to that incorporating the CAAAC motif positioned *N*-terminally may be a very efficient method for simultaneously inducing dimerization and α -helical structure of peptides by simple addition of Ag⁺.

Conclusions

It was demonstrated that coordination of Ag⁺ to CXXXC peptides such as Ac-RCAAAC-NH₂ (1) causes a change in secondary structure from random coil to a folded species, with a maximum α -helical signal observed using 2 equiv. of Ag⁺. The helical folding of 1 in the presence of Ag⁺ displayed a remarkable stability with only a slight decrease in folding below pH 3 and above pH 8 as well as persisting at physiological chloride levels. A series of titration, CD, NMR and LC-ESI-MS data support that the $\alpha\text{-helical complex}$ is a dimeric peptide species with four Ag⁺ bound. However, at low pH one Ag⁺ may be substituted for a proton. Potentiometric analysis confirmed that such multinuclear oligomeric species may form in solution at both low and neutral pH. N-Terminal incorporation of the motif CAAAC in a longer peptide caused significant propagation of α -helicity in response to Ag⁺. Overall, the silver coordination of the CXXXC motif presents an intriguing case of stable, metalinduced α -helical peptide folding with possible biomolecular and biomedical applications. Hence, incorporation of this motif can be a generally applicable tool for controlling peptide folding and assembly under acidic to neutral pH conditions. The control of peptide oligomerization state has great significance for both naturally occurring peptides and proteins as well as their synthetic counterparts e.g. biologics and peptide-based drugs.

Supporting Information

Detailed experimental procedures, compound data, circular dichroism spectra, analysis of pH-potentiometric data, HPLC chromatograms, LCMS data and NMR spectra are provided in the Supporting Information. The authors have cited additional references within the Supporting Information.^[30-38]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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