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Metal Ion-Assembled Micro-Collagen Heterotrimers

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Abstract

Collagen mimetic peptides (CMPs) provide critical insight into the assembly, stability and structure of the triple helical collagen protein. The majority of natural fibrous collagens are *aab* or *abc* heterotrimers, yet few examples of heterotrimeric CMPs have been reported. Previously CMP heterotrimers have only been accessible by total syntheses or by introducing complementary interstrand electrostatic or steric interactions. Here we describe an *abc* CMP heterotrimer in which each contributing CMP consists of only three amino acids: glycine, proline and 4-hydroxyproline. Assembly of the heterotrimeric triple helix is directed by a combination of metal-ion coordination to set the relative register of the CMPs, and minimization of valence frustration to direct heterotrimerization. Assembly of the four-component mixture is facile and extremely rapid, and equilibration to the *abc* heterotrimer occurs within a few hours at modestly elevated temperatures. The melting temperatures of the metal-assembled collagen trimers are higher by some 30 °C than the apo-peptide assemblies. Two iterations of the design are described, and the outcomes suggest possibilities for designing self-assembling *abc* and *abb* heterotrimers.

Introduction

Collagen is the most abundant animal protein and performs numerous structural roles. In addition to being the dominant component of cartilage, collagen has been implicated in directing bone growth and in critical signaling interactions necessary for tissue remodeling. Collagen is a multimeric fibrous protein hierarchically assembled from single polypeptide chains rich in the amino acids proline and glycine. Of 28 members of the collagen superfamily, the most prevalent are types I, II, III, V, and XI, all of which are fibril-forming proteins.¹ After extracellular extrusion and assembly, natural collagen is covalently cross-linked by a variety of spontaneous and enzyme-catalyzed processes. Consequently, natural collagen cannot be used as a source of collagenous peptides because cleavage of the cross-links is necessarily concomitant with cleavage of the peptide backbone. Synthetically tractable collagen mimetics are therefore required to create novel biomaterials, to enable the study of natural collagen assembly, and to explore biologically important protein-collagen interactions. Ideally such mimetics should be structurally well-defined and easy to manipulate.

Collagen consists of trimeric fibrils, each polypeptide chain of which favors a left-handed polyproline type II (PPII) geometry. In marked contrast to fibrous proteins composed of α -helical polypeptides, collagen trimers adopt an out-of-register arrangement of their constituent chains; for a canonical PPII sequence (POG)_n where P is L-proline, O is (2*S*, 4*R*)-4-hydroxyproline, and G is glycine, the P of chain *a* will be in register with the O of

chain *b* and the G of chain *c*. Single peptide chains are thus predisposed to associate as staggered right-handed supercoiled parallel trimers. Simple chains of repeating POG sequences adopt the natural staggered supercoil, but as collagen mimetics they are difficult to work with; shorter sequences are largely dissociated in aqueous media at physiological pH,² and longer sequences require prolonged annealing treatments before they adopt a unique, stable structure.³ The concept of constraining the contributing helices to overcome the tendency of short sequences to dissociate under physiologically relevant conditions has been advanced using a variety of creative approaches. Covalent templating strategies have been adopted to constrain the contributing CMPs to the desired trimeric topology⁴. Indeed, covalent homo- and heterotrimers have been assembled in a fragment condensation approach.⁵ Non-covalent chain elongation has been accomplished by engineering electronically complementary electron-rich and electron-poor p-systems at the N- and C-termini of short (32 residue) CMPs.⁶ Heterotrimeric self-assembly of single chain CMPs has been demonstrated by exploiting complementary interchain electrostatic⁷ and steric⁸ interactions between the PPII partners. Finally, trimer formation has been directed by installing metal-binding ligands into CMPs such that addition of appropriate metal ion imposes a register and oligomerization state predetermined by the requirements of the metal-ligand complex.⁹ To date, metal-assembled systems have been limited to the assembly of homotrimeric CMPs, which are necessarily sterically compromised at the metal center (if the CMPs are staggered), or must fail to adopt the desired interchain register if the geometric requirements of the metal center are satisfied. A notable variation on the metal-assembled collagen theme is Chmielewski's approach, wherein the metal does not directly assist trimer formation; rather metal-ligand coordination facilitates chain elongation and inter-trimer cross-linking.¹⁰

Clearly the optimal system is one that minimizes synthetic effort while also returning the target architecture with high confidence. Self-assembled systems, if they can be realized, allow these goals to be met. Assembly of a 1:1:1 heterotrimer must overcome the combinatorial entropy inherent in forming one of ten possible associations and which, in the absence of tailored interchain interactions, has only interchain H-bonding to provide the necessary free energy. For natural collagen, the interchain contribution is estimated to be 1.4 kcal/mol per H-bond.¹¹ The minimum difference in stability between an ideal in-register collagen heterotrimer with minimal valence frustration and the analogous homotrimer with an average of 2.67 H-bonds to water is therefore 3.7 kcal/mol (the number of H-bonds is an average: POG repeats have 4 unsatisfied H-bonds, GPO and OGP repeats each have 2 unsatisfied H-bonds). The entropy of mixing for three components that can form ten different trimeric associations is 4.30 cal/molK. Reduction to a single component thus costs 1.3 kcal/mol at 298 K, which is easily compensated by the estimated stability difference. Self-selection of 1:1:1 heterotrimers with minimal valence frustration is thus thermodynamically favorable, and the challenge is to capture the favored arrangement. Consequently we describe here a metal-directed assembly of heterotrimeric CMPs in which the contributing peptides are designed for energetically cooperative interactions between metal ion binding and out-of-register collagen triple helix formation. As a first step towards the design of self-assembled supramolecular collagen systems of predictable size, we have determined the extent to which the natural, out of register collagen pattern self-selects under the direction of metal-ligand complex formation. We have discovered that the distribution of species in these designed microcollagen metalloptides can be dominated by subtle effects; in particular the amino acid sequence of the distal dipeptide (the C-terminal amino acids, furthest from the metal center) provides an unexpected bias. We demonstrate that this bias can be corrected by re-engineering the C-terminus of one of the CMP sequences, resulting in high selectivity for the metal-assembled 1:1:1 heterotrimer.

In our initial studies three peptides, **c1**, **c2** and **c3** were designed and synthesized (Figure 1). The sequences consist of 5 repeats of the POG tripeptide. Additional residues were appended to the N- and C-termini to minimize valence frustration in the desired **c1c2c3** heterotrimer. Bidentate 2,2'-bipyridyl ligands were designed to be optimally placed for formation of the **c1c2c3** heterotrimer upon addition of an octahedral coordinating metal ion. The metal tris-bipyridyl complex is thus expected to set the register of the trimer as modeled in Figure 2. Nickel(II) was the metal of choice for triggering the assembly of microcollagen metallopolymers. Iron(II) has previously been adopted for similar roles in directed polypeptide assembly,^{9a,11} but it is susceptible to irreversible oxidation at the elevated temperatures required for thermal unfolding experiments. Polypyridyl complexes of nickel(II) are stable, and have the necessary kinetic lability to allow the ligand exchange necessary for establishing an equilibrium population of metallotrimers.

Materials and Methods

General

All reagents and protected amino acids were obtained from Sigma-Aldrich (St. Louis, MO), Advanced Chemtech (Louisville, KY), Novabiochem (La Jolla, CA), or ChemImpex (Wood Dale, IL) and used without further purification. Isotopically labeled proline-d7 was purchased from Cambridge Isotope Laboratories (Andover, MA) as the free amino acid and protected according to literature procedures.¹³ N,N-Dimethylformamide (DMF) was dried over freshly activated 4Å molecular sieves for at least 12 h before use. N,N-Diisopropylethylamine (DIEA) was distilled from ninhydrin under N₂, then redistilled from anhydrous NaOH under N₂. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analyses were performed on a Voyager DE-PRO spectrometer (AB Sciex, Foster City, CA) using dihydroxybenzoic acid as the matrix.

Peptide Synthesis

All peptides were synthesized on a 25 µM scale using standard solid-phase Fmoc-mediated protocols.¹⁴ Automated peptide synthesis was performed on an Advanced Chemtech 496 Omega 96-well multiple peptide synthesizer (Louisville, KY). MBHA polystyrene resin at 0.2 mmol/g loading (Novabiochem, La Jolla, CA) was used as the support. Fmoc deprotection was effected by 4-methylpiperidine (30 and 15% by volume sequentially) in DMF. Amino acid coupling was effected using O-benzotriazole-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HBTU, 0.25 M) in DMF. Eight molar equivalents of Fmoc-protected amino acids were used per coupling. Glycine residues were double coupled. 2,2'-Bipyridyl-5-carboxylic acid¹⁵ was appended to the deprotected N-terminus of the peptide by activation with HBTU (0.95 eq.) and DIEA (4 eq) in DMF over 1 h. Resin-bound peptides were washed with dry dichloromethane (DCM) and side-chain protecting groups were removed by treatment with 1,1,1-trifluoroacetic acid (TFA, 2 × 1 min). Peptides were cleaved from the support by treatment with anhydrous HF (10 mL, 45 min at 0 °C) with 10 % v/v anisole as scavenger. After evaporation of HF, the residue was suspended in dry ether, filtered, and washed with dry ether. The solids were resuspended in acetic acid (5% v/v, 50 mL), filtered to remove the resin, and the filtrate was lyophilized to dryness. The crude peptides were dissolved in deionized water and purified by reverse-phase HPLC with a Proteo-200 C-18 column (Higgins Analytical, Mountain View, CA) using a mixture of water (0.1% TFA) and acetonitrile (0.1% TFA) as a mobile phase. The eluent stream was monitored by UV-visible absorbance at 230 nm. The purified peptides were analyzed by MALDI-TOF-MS: **c1** *m/z* calc. for MH⁺ 1803.9, obs. 1803.8; **c2** *m/z* calc. for MH⁺ 1746.9, obs. 1746.5; **c3** *m/z* calc. for MH⁺ 1763.9, obs. 1764.0; **c1*** *m/z* calc. for MH⁺ 1759.9, obs. 1759.7; **c3**(proline-d₇) *m/z* calc. for MH⁺ 1769.9, obs. 1769.6.

Nickel(II) complexes

Solutions of lyophilized peptides (1 – 5 mM, 250 μ L) were prepared in ddH₂O resulting in solutions of pH $\sim$ 3. A solution of NiCl₂ (0.333 mol equivalent in ddH₂O) was added, and complex was allowed to form over 30 min. Exchange into buffer for spectroscopy (25 mM NaOAc, pH 6) or ESI-MS (5 mM NH₄OAc, pH 6) was accomplished by gel filtration (PD 10, GE Healthcare, Uppsala, SE). The resulting solutions are stable in air. The concentrations of the solutions were determined from the absorbance at 303 nm (ϵ_{303} = 39,400 M⁻¹cm⁻¹).

Thermal unfolding studies

Circular dichroism (CD) spectroscopy was performed on a Jasco 815 spectropolarimeter (Jasco, Easton, MD) equipped with PFD425S Peltier accessory. CD spectra were acquired in 1 mm cells (Hellma USA, Plainview, NY) using a 4 nm bandwidth and a scan rate of 0.5 nm/s. CD spectra were recorded at temperature increments of 2 °C from 5 °C to 85 °C with an equilibration time of 10 min at each step. Identical curves were obtained at longer (40 min) equilibration times. Peptide concentrations were 100 μ M (Ni(II) trimer concentrations were 33 μ M). Samples were stored at 4°C for at least 24 h, and were degassed and purged with N₂ before analysis. Singular value decomposition of the matrix of spectra **D** (wavelength vs. ellipticity vs. temperature) into orthogonal basis vectors **U**, a diagonal matrix of singular values **S**, and a unitary row matrix of weighting coefficients **V**, indicated that > 90% of the data could be synthesized from two principal components, consistent with a two-state unfolding process.^{4f} UV-visible spectra obtained simultaneously with the CD spectra showed that the [Ni(bpy)₃]²⁺ complexes remained intact throughout the unfolding process. The concentration of free CMP was negligible and the unfolding equilibrium was best described by the two-state interconversion of folded metallotrimer and unfolded metallotrimer. The first principal vector of the matrix product **SV**^T (the reduced data) was fitted to the Gibbs-Helmholtz function as follows:

For the folding equilibrium

The equilibrium constant at a temperature T is given by

$$K_T = [F]/[U]$$

where

$$\ln K_T \frac{\Delta H}{R} \left(\frac{1}{T_m} - \frac{1}{T} \right) + \frac{\Delta C_p}{R} \left(\frac{T_m}{T} + \ln \frac{T}{T_m} - 1 \right)$$

SVD was performed using *MATLAB* (R2010b, MathWorks, Natick, MA). Fitting was performed using Levenburg-Marquardt non-linear least squares minimization with ΔH , and T_m as variable parameters. ΔC_p was fixed at 13 cal/molK.¹⁶

ESI mass spectrometry

Mass spectrometry of nickel(II) complexes was performed by direct infusion on an ABI 4000 QTRAP (AB Sciex, Foster City, CA). Samples (150 μ M in peptide, 50 μ M in nickel trimer) were sprayed from 5 mM aqueous ammonium acetate (TurboV source at 25 μ L/min). Optimal spectra were obtained from Q1 scans at low (20V) declustering potential. No uncomplexed peptides were observed under these conditions. Declustering potentials in excess of 100V induced dissociation of the complexes. Data were analyzed using *MATLAB*. The distribution of Ni(II) metallotrimers was modelled using a sum of synthetic isotope envelopes.¹⁷ Non-linear least squares optimization was performed using an implementation of the Broyden-Fletcher-Goldfarb-Shanno algorithm.¹⁸

Xray Crystallography

Crystals of **c2** were grown from saturated sodium hydrogen carbonate solution by hanging drop evaporation at room temperature. The initial peptide concentration was 10 mg/mL. The peptides crystallized as clear needles, and a single crystal 0.8 mm $\times$ 0.1 mm $\times$ 0.1 mm was mounted in a glass capillary. The structure was solved by molecular replacement using *PHASER*,¹⁹ starting from an idealized model lacking the bipyridyl groups. Bipyridyl groups were built into the model using 2F_o-F_c difference maps. Details are described in the Supporting Information.

Results and Discussion

Structure and Stability of CMP Apopeptides

CMP **c2** serendipitously crystallized as needles from sodium bicarbonate solution. Neither **c1** nor **c3** could be crystallized from any of 96 buffer/precipitant combinations. It appears from the x-ray crystal structure (Figure 3) that the added flexibility provided by the **c2** N-terminal gly-gly repeat allows π -stacking between the bipyridyl residues, augmenting the crystal packing. Such an arrangement is not accessible to **c1** or **c3**. The crystal structure of **c2** reveals the characteristic trimeric collagen helix with a 7/2 pitch, together with an interchain H-bonded network of water molecules very similar to that reported by Brodsky.²⁰ Other features such as C γ -endo pucker at proline and C γ -exo pucker at hydroxyproline are typical of other CMP structures. The crystal packing and inter-trimer distance of 14 Å (Figure 3b) are reminiscent of the quasi-hexagonal packing of natural collagen fibrils.²¹

The CD spectra of CMPs **c1**, **c2**, and **c3** in the absence of metal are concentration dependent and the 225 nm feature characteristic of the collagen triple helix is fully formed at concentrations above 100 μ M (Figure 4a). All three apo-peptides show cooperative thermal unfolding transitions as judged by changes in their CD spectra (Figure 4b). The melting temperatures are modest, and similar to those reported by other groups for similar length (POG)_n sequences.^{4b,4m,5,22} Uncertainties in the oligomerization states preclude any determination of thermodynamic folding parameters.

Stability, Structure, and Selection Preferences among Ni(II)-assembled CMPs

Addition of Ni(II) to the individual CMPs results in immediate metallotri-mer formation as judged by the appearance within seconds of the characteristic split [Ni(bpy₃)]²⁺ ligand-centered π - π^* absorbance peak centered on 303 nm. Assembly occurs at room temperature at physiological pH by simply adding an aqueous solution of NiCl₂ to a solution of CMP. Ni:**c1**₃ and Ni:**c2**₃ both show induction of Δ stereochemistry at the metal center as indicated by the feature at 300 nm (a ligand-centered π - π^* transition²³), and expected for a right-handed collagen supercoil. Ni:**c3**₃ unexpectedly shows significant Λ stereochemistry at the metal center. Measurement of the CD spectra of dilutions of the homotrimer complexes confirmed that all the homotrimeric complexes were > 90% intact at concentrations above

10 μ M. Thermal unfolding curves are presented in Figure 5, and thermodynamic parameters extracted by fitting to a two-state folding model are presented in Table 1. As expected, metal ion complexation increases the melting temperature of all the complexes compared to the apo-peptides, and stabilization occurs with an increase in collagen helix content. For the nickel(II) homotrimers the heating and cooling curves are superimposable, with no detectable hysteresis. UV-visible spectra obtained simultaneously with the CD data indicate that the metal ion is retained at all temperatures, although the chiral configuration at the metal center is lost as the peptides unfold. Inspection of the melting temperatures shows that the Ni:c1₃ and Ni:c2₃ complexes are somewhat more stable than the Ni:c3₃ homotrimer. The Ni:c1:c2:c3 heterotrimer was prepared by annealing an equimolar mixture of nickel(II) homotrimers overnight at 50 °C. The melting temperature of the annealed mixture is some 2 °C higher than the average of the homotrimers, but is significantly (5 °C) lower than the T_m for the Ni:c1₃ homotrimer. It should be noted that we have made no direct measurements of the thermodynamic parameters of pure heterotrimers. The reported values of T_m and ΔH for heterotrimers were observed convolutions of the species present at equilibrium and thus describe relative stability trends between homo- and heterotrimer CMP assemblies.

Formation of the equilibrium mixture of trimers from an equimolar mixture of Ni(II) homotrimers was followed by electrospray ionization (ESI) mass spectrometry. The utility of soft ionization techniques in analyzing the composition of equilibrium distributions of exchange-labile metallo-peptide oligomers has previously been demonstrated.²⁴ At low declustering potentials (5 - 20 V) the trimeric nickel(II) complexes could be clearly observed with no detectable apo-peptide. Higher declustering potentials (100 – 200 V) induced dissociation of the complexes, and nickel(II)-associated dimers and monomers were observed. The metallo-peptides preferentially charge with ammonium ions, so the 5+ charged species shown in Figures 6 and 8 carry 3 ammonium ions in addition to the 2+ charge from the Ni²⁺ ion. Heterotrimer preferences among the CMPs were explored by mixing equimolar solutions of Ni:c1₃, Ni:c2₃ and Ni:c3₃ homotrimers and following the re-equilibration by ESI-MS (Figure 6). At room temperature exchange was slow, with a half-life of several days. However at 50 °C the exchange half-life was approximately 1.5 hr (Figure 6c). Figure 6a shows the redistribution of species over time at 50 °C. It is clear that the dominant product of this low-temperature annealing process is the desired Ni:c1:c2:c3 heterotrimer.

For an equimolar mixture of c1,c2,c3 and Ni²⁺ the statistically expected trimer distribution is 1:1:1:3:3:3:3:3:3:6 for Ni:c1₃ Ni:c2₃ Ni:c3₃ Ni:c1₂:c2 Ni:c1₂:c3 Ni:c1:c2₂ Ni:c1:c3₂ Ni:c2₂:c3 Ni:c2:c3₂ and Ni:c1:c2:c3 respectively. After equilibration for 12 h at 50 °C the observed distribution is approximately 2:1:1:4:3:2:1:2:3:9 (Figure 6b). Although the desired Ni:c1:c2:c3 heterotrimer dominates, there are significant contributions from other c1 rich sequences. We surmised that c1 contributes more stabilization due to reduced C-terminal fraying. The C-terminal pro-hyp-amide is more constrained than the C-termini of peptides c2 or c3 (gly-pro-amide and hyp-gly-amide respectively). To test this hypothesis we prepared a modified c1 peptide, c1* in which the terminal hydroxyproline was replaced by glycine, a negative design feature that would increase C-terminal flexibility, matching that of CMPs c2 and c3.

Stability, Structure, and Selection Preferences among a Second Generation of Ni(II)-assembled CMPs

The amino-acid sequences of peptides c2 and c3 were retained, and peptide c1 was modified with a terminal glycine residue, a O18G variation giving the c1* CMP. The thermal stabilities of the Ni(II) assembled homotrimers were determined in thermal unfolding experiments as shown in Figure 7.

It is clear that the Ni:**c1***₃ homotrimer is greatly destabilized with a T_m of 53.5 °C compared with T_m = 61.3 °C for the Ni:**c1**₃ homotrimer. The exchange kinetics and product distribution of the homotrimer mixture were followed by ESI-MS and are shown in Figure 8. CMP **c1*** has the same molecular mass as **c2**, so for the ESI-MS experiments an isotopically labeled variant of **c1*** was synthesized with two proline-*d*₇ residues, giving a molecular mass of 1759.9 Da. To ensure unique masses for all 10 metallotrimers it was further necessary to include a proline-*d*₇ residue in **c3**, resulting in a molecular mass of 1769.9 Da for this CMP. After only 5 min, much of the starting Ni:**c1***₃ homotrimer had already been repartitioned into Ni:**c1***₂:**c2** and Ni:**c1***₂:**c3** heterotrimers. Equilibration was essentially complete within 2 h at 50 °C (Figure 8c), and the product distribution was highly selective for the Ni:**c1***:**c2**:**c3** heterotrimer. After equilibration for 12 h at 50 °C the observed distribution was approximately 1:1:2:1:3:2:2:1:2:12 (Figure 8b), and the desired heterotrimer was present at over twice the statistical expectation. The thermal stabilities of the Ni(II) heterotrimers measured after annealing 12 h at 50 °C reveal a remarkable increase in both T_m and ΔH_u for the Ni:**c1***:**c2**:**c3** heterotrimer compared to the Ni:**c1**:**c2**:**c3** mixture (Figure 9). The Ni:**c1***:**c2**:**c3** melt is also much more cooperative than that for the Ni:**c1**:**c2**:**c3** mixture, suggesting a single dominant species, rather than the mixture of species known to be present after annealing in the Ni:**c1**:**c2**:**c3** mixture. Taken together, these results indicate that the negative redesign of **c1** was a successful strategy for suppressing undesired **c1**-rich species and increasing the contribution of the desired Ni:**c1***:**c2**:**c3** heterotrimer. Furthermore, thermal equilibration to the desired species occurs rapidly at modestly elevated temperatures to yield an extremely stable micro-collagen structure.

Measuring the equilibrium populations of metal-assembled CMPs provides a rich seam of data for future studies. For example, consideration of the CMP distributions among the trimers shown in Figures 6c and 8c reveals that in both cases the Ni:**c2**₂:**c3** heterotrimer dominates the Ni:**c2**:**c3**₂ heterotrimer by a factor of 2.5. This raises the interesting possibility of further redesign to favor selection of the Ni:**c2**₂:**c3** heterotrimer from a mixture of **c2**, **c3** and Ni(II). It is likely that **c3** suppression is linked with the tendency of the Ni:**c3**₃ homotrimer to adopt the unexpected Λ geometry at the metal center, and future studies will explore the influence of the constrained amide geometry at the 2,2-bipyridyl-5-carboxamide moiety.

Conclusion

As a first step towards the design and implementation of a predictable metal-assembled collagen network we have explored the intrinsic partnering preferences among staggered CMP triple helical bundles. We find that metal templating serves to define the CMP aggregation number, set the interhelical register between the contributing CMPs, and increase the thermal stability of the resulting trimers by some 30 °C. Furthermore, if the peptides contributing to a given metallotrimer are free to exchange with other sequences, the minimization of valence frustration (“sticky ends”) can be exploited to direct heterotrimeric assembly. The distribution of metalloheterotrimers in an equimolar mixture of CMPs is surprisingly sensitive to differences in structural rigidity between the participating peptides. Nevertheless consideration of the positive and negative design features results in a predominant *abc* heterotrimeric collagen helix of high thermal stability without the need for complementary electrostatic or steric interactions. The resulting metal-assembled microcollagen heterotrimer can be rapidly and selectively assembled by annealing for 2 h at 50 °C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Myllyharju J, Kivirikko KI. *Ann Med.* 2001; 33:7–21. [PubMed: 11310942]
2. Sakakibara S, Inouye K, Shudo K, Kishida Y, Kobayashi Y, Prockop DJ. *Biochim Biophys Acta.* 1973; 303:198–202. [PubMed: 4702003]
3. Engel J, Bächinger HP. *Top Curr Chem.* 2005; 247:7–33.
4. (a) Goodman M, Feng Y, Melancini G, Taulane JP. *J Am Chem Soc.* 1996; 118:5156–5157.(b) Feng Y, Melancini G, Taulane JP, Goodman M. *J Am Chem Soc.* 1996; 118:10351–10358.(c) Feng Y, Melancini G, Taulane JP, Goodman M. *Biopolymers.* 1996; 39:859–872. [PubMed: 8946805] (d) Kwak J, Jefferson EA, Bhumralkar M, Goodman M. *Bioorg Med Chem.* 1999; 7:153–160. [PubMed: 10199665] (e) Kwak J, De Capua A, Locardi E, Goodman M. *J Am Chem Soc.* 2002; 124:14085–14091. [PubMed: 12440907] (f) Horng JC, Hawk AJ, Zhao Q, Benedict ES, Burke SD, Raines RT. *Org Lett.* 2006; 8:4735–4738. [PubMed: 17020290] (g) Rump ET, Rijkers DTS, Hilbers HW, de Groot PG, Liskamp RMJ. *Chem Eur J.* 2002; 8:4613–4621. [PubMed: 12362398] (h) Germann HP, Heidemann E. *Biopolymers.* 1988; 27:157–163. [PubMed: 3342275] (i) Fields CG, Lovdahl CM, Miles AJ, Matthias Hagen VL, Fields GB. *Biopolymers.* 1993; 33:1695–1707. [PubMed: 8241428] (j) Fields CG, Mickelson DJ, Drake SL, McCarthy JB, Fields GB. *J Biol Chem.* 1993; 268:14153–14160. [PubMed: 8314781] (k) Fields CG, Grab B, Lauer JL, Fields GB. *Anal Biochem.* 1995; 321:57–64. [PubMed: 8678321] (l) Hojo H, Akamatsu Y, Yamauchi K, Kinoshita M, Miki S, Nakamura Y. *Tetrahedron.* 1997; 53:14263–14274.(m) Barth D, Kyrielis O, Frank S, Renner C, Moroder L. *Chem Eur J.* 2003; 9:3703–3714. [PubMed: 12898697] (n) Koide T, Nishikawa Y, Takahara Y. *Bioorg Med Chem Lett.* 2004; 14:125–128. [PubMed: 14684312] (o) Slatter DA, Foley LA, Peachey AR, Nietlispach D, Farndale RW. *J Mol Biol.* 2006; 359:289–298. [PubMed: 16631195] (p) Kotch FW, Raines RT. *Proc Natl Acad Sci USA.* 2006; 103:3028–3033. [PubMed: 16488977]
5. Li Y, Mo X, Kim D, Yu SM. *Biopolymers.* 2011; 95:94–104. [PubMed: 20740489]
6. Cejas MA, Kinney WA, Chen C, Leo GC, Tounge BA, Vinter JG, Joshi PP, Maryanaff BE. *J Am Chem Soc.* 2007; 129:2202–2203. [PubMed: 17269769]
7. (a) Gauba V, Hartgerink JD. *J Am Chem Soc.* 2007; 129:2683–2690. [PubMed: 17295489] (b) Dong H, Paramonov SE, Hartgerink JD. *J Am Chem Soc.* 2008; 130:13691–13695. [PubMed: 18803383] (c) Fallas JA, Gauba V, Hartgerink JD. *J Biol Chem.* 2009; 284:26851–26859. [PubMed: 19625247]
8. (a) Slatter DA, Miles CA, Bailey AJ. *J Mol Biol.* 2003; 329:175–183. [PubMed: 12742026] (b) Hodges JA, Raines RT. *J Am Chem Soc.* 2005; 127:15923–15932. [PubMed: 16277536]
9. (a) Koide T, Yuguchi M, Kawakita M, Konno H. *J Am Chem Soc.* 2002; 124:9388–9389. [PubMed: 12167030] (b) Cai WB, Kwok SW, Taulane JP, Goodman M. *J Am Chem Soc.* 2004; 126:15030–15031. [PubMed: 15547991]
10. (c) Przybyla DE, Chmielewski J. *J Am Chem Soc.* 2008; 130:12610–12611. [PubMed: 18763780] (d) Pires MM, Chmielewski J. *J Am Chem Soc.* 2009; 131:2706–2712. [PubMed: 19182901] (e) Pires MM, Przybyla DE, Chmielewski J. *Angew Chem Int Ed.* 2009; 48:7813–7817.
11. Boryskina OP, Bolbukh TV, Semenov MA, Gasan AI, Maleev VY. *J Mol Struct.* 2007; 827:1–10.
12. (a) Lieberman M, Tabet M, Sasaki T. *J Am Chem Soc.* 1994; 116:5035–5044.(b) Cuenoud B, Schepartz A. *Science.* 1993; 259:510–513. [PubMed: 8424173] (c) Ghadiri MR, Soares C, Choi C.

- J Am Chem Soc. 1992; 114:825–831.(d) Lieberman M, Sasaki T. J Am Chem Soc. 1991; 113:1470–1471.(e) Roy L, Case MA. Chem Commun. 2009:192–194.
13. Carpino LA, Han GY. J Org Chem. 1972; 37:3404–3409.
14. Wellings DA, Atherton E. Methods Enzymol. 1997; 289:44–67. [PubMed: 9353717]
15. Kröhnke F. Synthesis. 1976:1–25.
16. Doi M, Nishi Y, Uchiyama S, Nishiuchi Y, Nishio H, Nakazawa T, Ohkubo T, Kobayashi Y. J Pept Sci. 2005; 11:609–616. [PubMed: 15880478]
17. Rockwood AL. Rapid Commun Mass Spectrom. 1995; 9:103–105.
18. Kroon, DJ. 2009.
<http://www.mathworks.com/matlabcentral/fileexchange/23245-fminlbfgs-fast-limited-memory-optimizer>
19. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. J Appl Cryst. 2007; 40:1658–674.
20. Bella J, Eaton M, Brodsky B, Berman HM. Science. 1994; 266:75–81. [PubMed: 7695699]
21. (a) Orgel JP, Irving TC, Miller A, Wess TJ. Proc Natl Acad Sci USA. 2006; 103:9001–9005. [PubMed: 16751282] (b) Hulmes DJ, Miller A. Nature. 1979; 282:878–880. [PubMed: 514368]
22. (a) Bretscher LE, Jenkins CL, Taylor KM, DeRider ML, Raines RT. J Am Chem Soc. 2001; 123:777–778. [PubMed: 11456609] (b) Shah NK, Ramshaw JAM, Kirkpatrick A, Shah C, Brodsky B. Biochemistry. 1996; 35:10262–10268. [PubMed: 8756681] (c) Heidemann E, Roth W. Adv Polym Sci. 1982; 43:143–203.(d) Henkel W, Vogl T, Echner H, Voelter W, Urbanke C, Schleuder D, Rauterberg J. Biochemistry. 1999; 38:13610–13622. [PubMed: 10521268] (e) Tanaka Y, Suzuki K, Tanaka T. J Pept Res. 1998; 51:413–419. [PubMed: 9650715]
23. Wada A, Katayama C, Tanaka J. Acta Cryst. 1976; B32:3194–3199.
24. (a) Cooper HJ, Case MA, McLendon GL, Marshall AG. J Am Chem Soc. 2003; 125:5331–5339. [PubMed: 12720445] (b) Telfer SG, Yang XJ, Williams AF. Dalton Trans. 2004:699–705. [PubMed: 15252489] (c) Roy L, Case MA. J Am Chem Soc. 2010; 132:8894–8896. [PubMed: 20536133]

FIGURE 1.
Peptide sequences used in this study.

FIGURE 2.
Computer-generated model of the Ni(II)-assembled Ni:**c1:c2:c3** heterotrimer.

FIGURE 3.

X-ray crystal structure of **c2** homotrimer (a) $2F_o - F_c$ map (mesh) and atomic structure (sticks) of the **c2** homotrimer. (b) View along the crystallographic a-axis showing the pseudo-hexagonal packing of the homotrimers. Center-to center inter-trimer spacing is 14 Å.

FIGURE 4.

(a) CD spectra and (b) thermal melts of CMPs **c1** (red), **c2** (green), **c3** (blue), and an equimolar mixture of **c1**, **c2**, and **c3** (black). Spectra were recorded at 20 °C, 100 μM peptide concentrations, and are corrected for 17 contributing residues. Melts were recorded at 100 μM trimer concentration and the data has been scaled to the CD signal at 225 nm.

FIGURE 5.

(a) CD spectra and (b) thermal melts of Ni(II) complexes of CMPs Ni:c1₃ (red), Ni:c2₃ (green), Ni:c3₃ (blue), and an equimolar mixture of Ni:c1₃, Ni:c2₃, and Ni:c3₃ (black). Spectra were recorded at 20 °C, 33 μM trimer concentrations, and are corrected for 51 contributing residues. Melts were recorded at 33 μM trimer concentration and the data has been scaled to the CD signal at 225 nm. The Ni:c1₃, Ni:c2₃, Ni:c3₃ mixture was annealed overnight at 50 °C before measuring the unfolding.

FIGURE 6.

Establishment of the equilibration distribution of a mixture of Ni(II) heterotrimers. (a) ESI-MS spectra recorded at 5 – 720 min after mixing an equimolar mixture of Ni:c1_3 , Ni:c2_3 and Ni:c3_3 homotrimers at 50 °C. The $m/5z$ charge state is shown. The charge on the complex is provided by the Ni^{2+} ion and 3 NH_4^+ ions from the ammonium acetate buffer. Consequently $[\text{Ni:c1}_3]^{2+} \cdot 3\text{NH}_4^+$ has a mass of 5521.5 Da and the $m/5z$ signal is calculated to be 1104.3 Da. The calculated $m/5z$ masses for $[\text{Ni:c2}_3]^{2+} \cdot 3\text{NH}_4^+$ and $[\text{Ni:c3}_3]^{2+} \cdot 3\text{NH}_4^+$ are 1070.1 Da and 1080.3 Da respectively. The time axis is on a logarithmic scale for easier viewing. (b) Fitted isotope envelopes for the first (5 min, blue) and last (720 min, red) mass spectra. From left to right the species are Ni:c2_3 , $\text{Ni:c2}_2\text{:c3}$, Ni:c2:c3_2 , Ni:c3_3 , Ni:c1:c2_2 , Ni:c1:c2:c3 , Ni:c1:c3_2 , $\text{Ni:c1}_2\text{:c2}$, $\text{Ni:c1}_2\text{:c3}$, Ni:c1_3 . (c) Time course for the equilibration process at 40 °C (filled circles) and 50 °C (empty circles).

FIGURE 7.

(a) CD spectra and (b) thermal melts of Ni(II) complexes of CMPs Ni:c1*₃ (red), Ni:c2₃ (green), Ni:c3₃ (blue), and an equimolar mixture of Ni:c1*₃, Ni:c2₃, and Ni:c3₃ (black). Spectra were recorded at 20 °C, 33 μM trimer concentrations, and are corrected for 51 contributing residues. Melts were recorded at 33 μM trimer concentration and the data has been scaled to the CD signal at 225 nm. The Ni:c1*₃, Ni:c2₃, Ni:c3₃ mixture was annealed overnight at 50 °C before measuring the unfolding.

FIGURE 8.

Establishment of the equilibration distribution of a mixture of second generation Ni(II) heterotrimers. (a) ESI-MS spectra recorded at 5 – 720 min after mixing an equimolar mixture of Ni:**c1***₃, Ni:**c2**₃ and Ni:**c3**₃ homotrimers at 50 °C. The m/5z charge state is shown. To ensure unique masses for all ten possible combinations, **c1*** contains two proline-*d*₇ deuterated residues and **c3** contains one proline-*d*₇ residue. Consequently [Ni:**c1***₃]²⁺.3NH₄⁺ has a mass of 5392.5 Da and the m/5z signal is calculated to be 1078.5 Da. The calculated m/5z masses for [Ni:**c2**₃]²⁺.3NH₄⁺ and [Ni:**c3**₃]²⁺.3NH₄⁺ are 1070.1 Da and 1084.5 Da respectively. The time axis is on a logarithmic scale for easier viewing. (b) Fitted isotope envelopes for the first (5 min, blue) and last (720 min, red) mass spectra. From left to right the species are Ni:**c2**₃, Ni:**c1***:**c2**₂, Ni:**c2**₂:**c3**, Ni:**c1***₂:**c2**, Ni:**c1***:**c2**:**c3**, Ni:**c1***₃, Ni:**c2**:**c3**₂, Ni:**c1***₂:**c3**, Ni:**c1***:**c3**₂, Ni:**c3**₃. (c) Time course for the equilibration process at 50 °C.

FIGURE 9.

Thermal melting curves corrected for baseline offsets. (a) Ni:c1₃ (red), Ni:c2₃ (green), Ni:c3₃ (blue), annealed mixture of Ni:c1₃, Ni:c2₃, and Ni:c3₃ (black). (b) Ni:c1*₃ (red), Ni:c2₃ (green), Ni:c3₃ (blue), annealed mixture of Ni:c1*₃, Ni:c2₃, and Ni:c3₃ (black).

TABLE I

Thermodynamic folding parameters extracted from fits to the Gibbs-Helmholtz equation.

Ni(II) CMP complex	ΔH_f (kcal/mol)	T_m (°C)	ΔG_f (kcal/mol) ^a
Ni:c1 ₃	-33.0	61.3	-3.6
Ni:c2 ₃	-21.4	58.4	-2.2
Ni:c3 ₃	-34.4	43.4	-2.0
Ni:c1* ₃	-24.2	53.3	-2.1
Ni:c1:c2:c3	-24.6 ^b	56.3 ^b	-2.3
Ni:c1*:c2:c3	-41.9 ^b	68.0 ^b	-5.3

^a ΔG_f is calculated from $\Delta G_f = \Delta H_f - 298.15\Delta H_f/(T_m + 273.15)$

^b Observed values of T_m and ΔH are convolutions of those parameters for the species present at equilibrium.