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Methods for Solving Highly Symmetric *de Novo* Designed Metalloproteins: Crystallographic Examination of a Novel Three Stranded Coiled Coil structure containing D-amino Acids

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Abstract

The core objective of *de Novo* metalloprotein design is to define metal-protein relationships that control the structure and function of metal centers by using simplified proteins. An essential requirement to achieve this goal is to obtain high resolution structural data using either NMR or crystallographic studies in order to evaluate successful design. X-ray crystal structures have proved that a four heptad repeat scaffold contained in the three stranded coiled coil (3SCC), called CoilSer, provides an excellent motif for modeling a three Cys binding environment capable of chelating metals into geometries that resemble heavy metal sites in metalloregulatory systems. However, new generations of more complicated designs that feature, for example, a D-amino acid or multiple metal ligand sites in the helical sequence require a more stable construct. In doing so, an extra heptad was introduced into the original CoilSer sequence, yielding a GRAND-CoilSer to retain the 3SCC folding. An apo-(GRAND-CSL12_DLL16C)₃ crystal structure, designed for Cd(II)S₃ complexation, proved to be a well-folded parallel 3SCC. Because this structure is novel, protocols for crystallization, structural determination and refinements of the apo-(GRAND-CSL12_DLL16C)₃ are described. This report should be generally useful for future crystallographic studies of related coiled coil designs.

Keywords

De Novo protein design; Three stranded coiled coil; D-amino acid; X-ray crystallography

INTRODUCTION

De novo designed proteins provide a simplified scaffold that allows us to construct a metal site to learn the basic principles of metal-protein interactions that are, often times, challenging to address in a natural protein system due to its high level of complexity.^{1–7} For decades, the Pecoraro group has been interested in studying heavy metal chemistry in proteins by introducing a tris-thiolate rich site into the hydrophobic core of a 3SCC scaffold based on the **TRI**-family peptide sequence (sequence shown in Table 1). The helix is designed based on a heptad repeat approach. Hydrophobic residues are placed at the *a* and *d*

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positions which undergo hydrophobic collapse when helices associate into a tertiary coiled coil structure in solution. Hydrophilic residues at *e* and *g* facilitate salt bridge interactions between strands to stabilize the 3SCC orientation at an appropriate pH. The replacement of a core Leu residue with a Cys yields a three Cys environment for heavy metal binding studies (Figure 1). Spectroscopic studies have shown that the engineered peptides are capable of direct binding to a variety of metals (Hg(II), Cd(II), Pb(II), As(III), Zn(II) and Bi(III)) in which the knowledge gained can be applied to investigate heavy metal interactions in the metalloregulatory systems.^{2,8–29} Despite the success of spectroscopic experiments from solution studies, structural details of the metal structures are needed in order to provide a thorough understanding of how these tris-thiolate environments lead to each specific metal coordination mode based on their geometric preferences. Unfortunately the TRI-family peptides were not capable of generating high quality protein crystals, therefore, a closely related CoilSer peptide has been used to serve as a crystallographic analogue. The CoilSer sequence was also designed based on a heptad repeat approach³⁰ and self-assemble into a parallel 3SCC in a similar manner to TRI when the Cys-rich site is incorporated.²³ Spectroscopic studies demonstrated that TRI and CoilSer derivatives binds to metals at the sulfur site in an analogous manner¹⁸; however, the presence of a His residue at the 28th position allows CoilSer to create a 3D packing of peptides by forming external Zn(II) sites between symmetry related trimers. X-ray crystal structures have been obtained for **CoilSer** peptides with thiol containing ligand substitution^{22,23,27,31,32}, however, these few structures do not allow one to assess the consequences of new features incorporated in more advanced protein designs where, for example, multiple metal sites are present or a drastic destabilizing residue is introduced into the parent sequence. Because the stability of these amphipathetic 3SCCs is dependent on exclusion of residues such as leucine from the aqueous environment, the number of unperturbed a and d hydrophobic layers determines the aggregate stability. Designs including multiple modifications in the hydrophobic interior are no longer stable with the relative short TRI scaffold so that GRAND peptides, containing an additional heptad needed to be developed. Herein, we report a GRAND-CoilSer in which an additional heptad is incorporated into the original CoilSer sequence to enhance the stability of the aggregates for crystallographic studies. The His residue, now placed at the 35th position (last heptad) of the elongating peptide, allows for high quality crystal formation. The designed sequence reported in this article is **GRAND-CSL12_DLL16C** where a D-Leu residue at the 12th position was designed to generate steric encumbrance above the Cys site to enforce an unusual Cd(II)S₃ center in a 3SCC environment.^{20,32} The **GRAND-CSL12_DLL16C** serves as a crystallographic analogue for **TRIL12_DLL16C**. Original structural studies were attempted using the **CSL12_DLL16C** derivative; however, crystals were not obtained due to the decreased stability associated with the packing of a D-Leu into the sequence.³² By adding an extra heptad, the resulting apo-(**GRAND-CSL12_DLL16C**)₃ crystal structure reveals a parallel 3SCC peptide resembling the previous Cys-rich CoilSer helices (Figure 2). The two types of peptides, however, were crystallized in different space groups in which the GRAND-CoilSer helices are packed in the high symmetry space group R32 that requires a crystallographically imposed threefold axis along the three helices, while the shorter CoilSer derivatives pack differently with the space group C2. A methodology for crystallization setup and structure determination is provided as this is the first time a **GRAND-CS** derivative is solved. Elongating the 3SCC illustrates how stable assemblies that cannot be

crystallized using shorter derivatives can be realized and illustrates the potential impact of D-amino acid chirality on controlling a metal center coordination. This approach emphasizes the benefits of the expansion of the heptad repeats to achieve sufficient overall stability in the coiled coil system when drastic mutations are included. This observation may open up new vistas for metalloprotein engineering that could greatly expand the structural understanding of new designed scaffolds for further biophysical, catalytic and therapeutic applications.

MATERIALS

Synthesis & Purification

Fmoc-protected amino acids and the MBHA rink amide resin from Novabiochem; *N*-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) from Anaspec Inc.; diisopropylethylamine (DIEA), acetic anhydride, piperidine and pyridine from Sigma Aldrich; *N*-methylpyrrolidinone (NMP), *N,N*-dimethylformamide (DMF), anisole, thioanisole, ethanedithiol, Trifluoroacetic acid and acetonitrile (HPLC grade) from Fisher Scientific.

Crystallization

Tris(hydroxymethyl)aminomethane and zinc acetate dehydrate (colorless solid) were purchased from Sigma Aldrich. PEG light screen was obtained from Hampton Research.

METHODS

1. Synthesis & Purification

The GRAND-CSL12_DLL16C peptide was synthesized on an Applied Biosystems 433A automated peptide synthesizer with Fmoc-protected amino acids using the standard Fmoc protocol (Applied Biosystems).³³ The C-terminus of the peptides was amidated on the solid support MBHA rink amide resin (0.25 mmole scale) with HBTU/HOBt/DIEPA coupling methods. The N-terminus was acetylated with a solution of 4% (v/v) acetic anhydride, 4.3% (v/v) pyridine, and 91.7% *N,N*-dimethylformamide (DMF). The peptide was cleaved from the resin using a cleavage mixture of 90% trifluoroacetic acid (TFA), 5% anisole, 3% thioanisole, and 2% ethanedithiol for 3.5 hours. The cleaved peptide solution was filtered and evaporated under a dry N₂-flow until a glassy film appeared on the surface. Cold diethyl ether was then added to the thin film to obtain a precipitated white crude peptide. This crude was re-dissolved in ddH₂O and lyophilized to get a fluffy white powder which was subsequently dissolved in 10% acetic acid.

2. Peptide Purification

The peptide was purified by reversed phase HPLC on a Waters 600 Semiprep HPLC peptide C-18 using a linear gradient of 0.1% TFA in water to 0.1% TFA in 9:1 CH₃CN/H₂O program over 30 mins (flow rate 10 mL/min). The purified peptides were identified by electrospray mass spectrometry. The actual mass of the GRAND-CSL12_DLL16C was determined to be 4,147.55 g/mol corresponding to the theoretical calculation.³⁴ The purified solution was frozen under liquid N₂ and lyophilized until the peptide was completely dry.

3. Protein Crystallization

About 25 mg of white power GRAND-CSL12_DLL16C peptide was dissolved in degassed 250 μ L ddH₂O as a peptide stock. The sample was prepared to yield the final concentration of 20 mg/mL peptide with a total volume of 420 μ L. Tris buffer at pH 8.5 was added to make 5mM concentration following by an addition of Zn(OAc)₂ solution to reach a final concentration of 15 mM Zn(OAc)₂. After every addition of a new reagent, the sample was vortexed and centrifuged at 14,000 rpm for a minute to ensure that the sample was well-dissolved.

The suitable condition for crystallization was setup robotically with a Crystal Gryphon machine from Art Robbins Instruments using a PEG light screen from Hampton Research. The screen was performed on a 96-well sitting drop plate with drops containing equal volumes of peptide (0.75 μ L) and precipitant (0.75 μ L) solutions. The crystals were grown by vapor diffusion at 20 °C. The appropriate precipitant condition for GRAND-CSL12_DLL16C crystallization contains 40% (v/v) PEG-400, sodium acetate buffer pH 4.5 at a final well solution pH 5.4. Crystals were cryoprotected in a mother liquor containing 20% glycerol prior to supercooling in liquid N₂ for data collection.

4. Data Collection and Processing

Data collection was performed at the Advanced Photon Source (APS) of the Argonne National Laboratory on the LS-CAT Beamline 21-ID-F equipped with a Mar 225 CCD detector at a wavelength of 0.97872 Å.³⁵ The data were collected with a 1° oscillation and 1s exposure time. The data were processed with HKL2000.³⁶ The first step of data processing involves the determination of the crystal system and unit cell dimensions. The GRAND-CSL12_DLL16C crystal was diffracted to 1.42 Å with the R32 (#155) space group. The unit cell parameters are shown in Table 2. The reflections of each image were indexed and assigned as integers: *h*, *k*, and *l*. The intensities of the reflections were measured and then scaled with SCALEPACK³⁶ so that the intensities of reflections from all images were related and represent the quality of the overall dataset (See Table 2 for data collection statistics.). The output file from the scaling contains the index of each spot and its measured intensity. This file was sorted so that the spots are listed in numerical order in .mtz format using the CCP4 suite of programs.^{37–39}

5. Structure Determination and Refinements

The structure determination process began with the estimation of the numbers of molecules in an asymmetric unit (ASU) using a method developed by Matthew.⁴⁰ The GRAND-CSL12_DLL16C, crystallized in the R32 space group, was determined to contain one single strand of the 3SCC peptide per ASU. The Matthew's coefficient of 2.38 corresponds to 47.67% solvent content. (The 3SCC is obtained by the combination of three adjacent symmetric units that are crystallographic imposed by the threefold axis.) This is in marked contrast with the previous CoilSer crystal structures where most were crystallized in the C2 space group (#5), consequently containing a single trimer per ASU.^{22,23,27,31,32} Due to the high sequence identity, the GRAND-CSL12_DLL16C structure was solved using a four-heptad chain A of the published 3SCC apo-(CSL9C)₃ (PDB code: 3LJM)²² to serve as a search model in the auto MolRep⁴¹ in the CCP4 suite of programs^{37–39}, in which the Cys9

and Leu16 were mutated to Leu and Cys, respectively. The phases calculated from the molecular replacement solution were then used in autobuilding Arp/warp software⁴² to generate a model that has five heptads in length corresponding to the **GRAND-CoilSer** structure. The percentage ratio of R/R_{free} that resulted from the autobuilding Arp/warp was 35.3/42.7. Then, the model underwent iterative rounds of electron density fitting and refining in Coot⁴³ and Buster 2.11.2 program⁴⁴, respectively. After the first round of rigid body refinement, the D-Leu residue (DLE) was built at the 12th position using the difference density present in $F_o - F_c$ map. A Zn(II) ion was constructed based on the $F_o - F_c$ map closed by the C-terminus of the structure. The refined metal site displays a tetrahedral geometry with 31Glu, 34Glu and 35His from the corresponding ASU and the fourth ligand from 3Glu of a symmetry related molecule. The site is located at the crystallographic interface to link the helices into the lattice form. Acylated and amidated groups were built to cap the N- and C-termini, respectively. A PEG chain and waters were added upon refinements. The validity of the model was verified using the MolProbity software.⁴⁵ All non-glycine residues of these structures fall in the preferred right handed α -helical region of the Ramachandran plot. Every side chain is present in the preferred rotameric conformations. The final model was refined to 1.42 Å ($R_{\text{working}} = 19.6\%$, $R_{\text{free}} = 20.3\%$). The 3SCC visualization was achieved through threefold symmetry related helices from three adjacent ASUs. The lack of $F_o - F_c$ electron difference for a metal at the Cys site (16th position) reveals the (GRAND-CSL12_DLL16C)₃ to be an apo-protein. The apo-(GRAND-CSL12_DLL16C)₃ is unperturbed by D-Leu as clearly demonstrated by the similarity in secondary structure of the helical backbones between the longer apo-(GRAND-CSL12_DLL16C)₃ and shorter apo-(CSL9C)₃ helices (Figure 3). Moreover, the major Cys conformations of apo-(GRAND-CSL12_DLL16C)₃ are similar to the apo-(CSL9C)₃ and the apo-(CSL16C)₃ indicating that the thiols rotamers in the *a* site of the 3SCCs, regardless of the length of the helices, are identical in the absence of metal. Therefore, the apo-(GRAND-CSL12_DLL16C)₃ crystal structure provides structural insight of elongating Cys-rich CoilSer derivative and represents a useful scaffold for future active site metalloprotein modelling.

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Figure 1.

PyMOL visualization demonstrating how to engineer a tris-thiolate binding site into the interior of 3SCC. The substitution of Leu residues (shown as pink sticks) with Cys yields three Cys environment that is capable of binding heavy metal atoms. The figure was made based on As(III)(CSL9C)₃ (PDB code: 2JGO).²³

Figure 2.

Ribbon diagrams of apo-(CSL9C)₃ (left) and apo-(GRAND-CSL12_DLL16C)₃ (right) representing the difference in length of the two peptides. The apo-(CSL9C)₃ contains four heptad repeats while an additional heptad is added in the apo-(GRAND-CSL12_DLL16C)₃ subsequently resulting in a longer trimer. Helical core residues are shown as sticks. D-Leu in the 12th position of apo-(GRAND-CSL12_DLL16C)₃ is red. Thiols of Cys residues are yellow.

Figure 3.

Side view of an overlay between the trimeric apo-(GRAND-CSL12_DLL16C)₃ and apo-(CSL9C)₃ structures indicating that there is no change in the overall secondary structures of GRAND-CoilSer and CoilSer derivatives. Moreover, the well-alignments demonstrates that the incorporation of a non-natural amino acid, D-Leu, does not perturb the secondary structure of the three-stranded coiled coil. Shown in green is the apo-(GRAND-CSL12_DLL16C)₃ and in pink is apo-(CSL9C)₃. Side chain residues are omitted for clarity.

Table 1

Peptide sequences

Peptides		a b c d e f g 2	a b c d e f g 9 12	a b c d e f g 16 19	a b c d e f g	a b c d e f g 30
TRI	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL12 _D LL16C	Ac-G	LKALEEK	LKALEEK	<u>C</u> KALEEK	LKALEEK	G-NH ₂
Coil-Ser (CS)	Ac-E	WEALEKK	LAALESK	LQALEKK	LEALEHG	-NH ₂
CSL16C	Ac-E	WEALEKK	LAALESK	<u>C</u> QALEKK	LEALEHG	-NH ₂
GRAND-CoilSer	Ac-E	WEALEKK	LAALESK	LQALEKK	LQALEKK	LEALEHG -NH ₂
GRANDCSL12 _D LL16C	Ac-E	WEALEKK	LAA <u>D</u> LESK	<u>C</u> QALEKK	LQALEKK	LEALEHG -NH ₂

Bold and underlined residues indicate substitutions.

C- and N-termini are capped by Ac and NH₂ groups, respectively.

Table 2

Crystallography Data Collection and Refinement Statistics.

Peptide	1SCC of GRAND-CSL12 _D LL16C (per ASU)
Data Collection	
Space Group	R32
Unit Cell a, b, c (Å)	38.213, 38.213, 140.655
Wavelength (Å)	0.97872
Resolution (Å) ¹	1.42(1.42–1.40)
R _{sym} (%) ²	5.6 (43.4)
<I/σI> ³	>50 (2)
Completeness (%) ⁴	99.3 (100)
Redundancy	5.6 (5.5)
Refinement	
Resolution (Å)	1.42
R-Factor (%) ⁵	19.6
R _{free} (%) ⁶	20.3
Protein atoms	302
Metal ions	Zn(II)
Water Molecules	52
Unique Reflections	8093
R.m.s.d. ⁷	
Bonds	0.010
Angles	1.15
MolProbity Score ⁸	1.11
Clash Score ⁸	3.17

¹ Statistics for highest resolution bin of reflections in parentheses.² $R_{\text{sym}} = \sum_h \sum_j |I_{hj} - \langle I_h \rangle| / \sum_h \sum_j I_{hj}$, where I_{hj} is the intensity of observation j of reflection h and $\langle I_h \rangle$ is the mean intensity for multiply recorded reflections.³ Intensity signal-to-noise ratio.⁴ Completeness of the unique diffraction data.⁵ $R\text{-factor} = \sum_h | |F_O| - |F_C| | / \sum_h |F_O|$, where F_O and F_C are the observed and calculated structure factor amplitudes for reflection h .⁶ R_{free} is calculated against a 10% random sampling of the reflections that were removed before structure refinement.⁷ Root mean square deviation of bond lengths and bond angles.⁸ Chen et al. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallographica D66:12–21.