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Laboratory Experiment

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Design and Characterization of a Zn^{2+}-Binding Four-Helix Bundle Protein in the Biophysical Chemistry Laboratory

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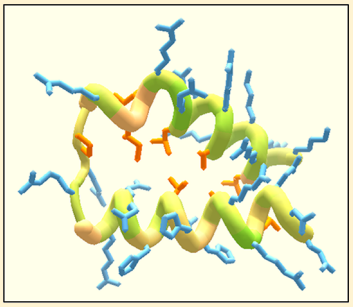
Supporting Information

ABSTRACT: A biophysical chemistry laboratory project is described that combines classroom and laboratory experiences to design and characterize a Zn^{2+}-binding four-helix bundle protein. The design phase involves re-examining principles of protein structure and function in the context of four-helix bundle literature and then using the online computer game Foldit to build and optimize a 35-residue helix-loop-helix peptide that would coordinate Zn^{2+}. The designed peptide and a control peptide containing the same amino acids but in a random sequence are purchased commercially. The peptides are then characterized by native polyacrylamide gel electrophoresis, immobilized-metal affinity chromatography, and ^{1}H NMR spectroscopy. The results indicate that the designed peptide adopts a higher-order structure than the control peptide, although not conclusively a four-helix bundle, and that it also binds Zn^{2+}.



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Computational design of proteins with desired catalytic or molecular-recognition properties has been an area of active research for more than a decade.[1−3] Design tools have the potential to impact the biotechnology and pharmaceutical industries significantly in the development of new biocatalysts, biosensors, diagnostic tests, and therapeutic agents.[4] A recent application of these methods was used to develop an antiviral protein directed against the H1N1 pandemic virus[5] and was discussed using an instructor-led journal club format in a prerequisite first-semester biochemistry course. Students thus began the project described here with a good understanding of amino acid structure, hydrophobic versus hydrophilic, the general features of protein structure, and the concept that structure is responsible for assembly and function. The pedagogical goals for this experiment are to integrate this information creatively to prospectively design a protein with a desired structure and function and to demonstrate several biophysical characterization methods. Previous laboratory experiments in this *Journal* have described protein folding[6] and secondary structure,[7] native polyacrylamide gel electrophoresis,[8,9] immobilized-metal affinity chromatography,[10,11] and NMR spectroscopy of peptides.[12,13] The experiment described here integrates these concepts and techniques into a design-synthesize-characterize multiweek project. Although the experiment described pertains to the four-helix bundle, the same methodologies could be applied for other small structural motifs, such as the leucine zipper or zinc finger.

The four-helix bundle is one of the early recognized recurring protein structural motifs.[14] It consists of four helices that pack together in antiparallel fashion connected by three loop regions, with the helices crossing each other at an angle of roughly 20°. As the helices diverge from their point of crossing, the surface area increases and binding sites are created that are specific to the role of the protein, such as binding to metal ions, cofactors, or other ligands. The tremendous sequence variability found in four-helix bundle proteins[15] is an excellent example of nature utilizing a common structural scaffold to present a wide diversity of functional surfaces. More recently, the propensity for even unevolved four-helix bundles to possess nascent ligand binding and/or enzymatic activity has been described.[16]

Common structural features contribute to the stability of four-helix bundle proteins despite the observed sequence diversity. The structural cores of the bundles that participate in interhelical packing are composed of close-packed, interdigitated nonpolar amino acids, whereas the connecting loops provide just enough flexibility for proper arrangement of the helices and diminish the entropic cost of bringing the four helices together. These structural features were identified more than 25 years ago and, in a series of elegant papers, were taken advantage of to design de novo four-helix bundle proteins.[17−20] It was ultimately shown that a peptide designed to consist of two identical 16-residue helix-forming segments separated by a three-residue loop dimerized in solution to form a four-helix bundle. This design was then extended to form Zn^{2+}-binding four-helix bundle by replacing three residues of the 35-residue peptide with histidine residues that would be positioned correctly in the three-dimensional motif to coordinate a zinc cation.

The four-helix bundle protein design project is introduced to students by using PyMol software[21] to visualize and explore the four-helix bundle proteins myohemerythrin[22] (PDB entry 2MHR) and apoferritin[23] (PDB entry 3RD0) interactively.

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These structures provide a context to discuss the experimental results on synthetic peptides and the resulting four-helix bundle design principles described above.[17−19,24] Concepts already learned in the prerequisite biochemistry course are re-examined in the context of these design principles. Subsequently, the structure of hemocyanin[25] (PDB entry 1LLA) is explored. Although not a four-helix bundle protein, the two Cu^{2+}-binding sites are each comprised of three histidine side chains extending from two antiparallel helices. This arrangement is mimicked in the design of the first Zn^{2+}-binding four-helix bundle[20] and provides the basis for the students' design, as well.

On the basis of the literature discussions and molecular visualizations, the students set forth the following design guidelines for their 35-residue peptide that would adopt a helix-loop-helix conformation, dimerize into a four-helix bundle, and bind to Zn^{2+}:

- amino acids should have a helical tendency
- each helix should have 16 residues
- helices should be separated with a three-residue loop
- helices should be amphiphilic
- helices should have glycine caps
- helices should contain some metal-coordinating amino acids such as histidine

EXPERIMENTAL DETAILS

Upper-division undergraduate biophysical chemistry meets weekly in a 150-min time-block allowing for a mixture of classroom and laboratory experiences. The four-helix bundle design project takes place during the first four and final two class periods of a 15-week semester. This allows time for procuring peptides while other topics are covered. Three class periods are spent discussing the four-helix bundle literature and introducing students to the online computer game Foldit[26,27] that is used to build and optimize a 35-residue peptide. Students first register as Foldit users and complete all tutorials through level 8 during class and as homework assignments. This allows students to become familiar with Foldit's capabilities and command interface. Several Foldit puzzles related to optimizing a protein's fold are also assigned as homework. This allows students to visualize concepts that had been discussed, while competing against themselves (and the instructor), as well as with other players all over the world. After mastering the tutorials and puzzles, students develop an intuitive feel for how Foldit scores correlate with the general principles of protein structure.

Students then spend one class period working in two small groups to design their peptide. After some time spent iterating the peptide sequence, taking into account all of the design principles simultaneously, the peptide sequence from the group with the highest Foldit score is declared the winner. The resulting 35-residue sequence was EDELRRLHHVEHEERGRRPGRIIRRLERVRKRLRK. The two designed helices (underlined) have different sequences and the three histidine residues are all located on the "outside" of the first helix. A BLAST search of the UniProt database indicated that this sequence was unique, with the best alignment having 60% identity. Students also designed a control peptide containing the same amino acids in a scrambled sequence. The sequence for the control peptide was EERLHEERRPRIREVKLKDLRHVHEGRGIRLRRRR.

Both peptides are purchased commercially through competitive bids; delivery time is four weeks. The peptides are characterized during the final two class periods using native polyacrylamide gel electrophoresis, immobilized-metal affinity chromatography, and NMR spectroscopy. Procedure details are found in the Supporting Information.

HAZARDS

Eye protection, gloves, and laboratory coats should be worn when preparing samples and when handling chemicals and reagents. Particular care should be taken when handling the precast polyacrylamide gels as they may contain unreacted acrylamide which is a neurotoxin. Electrophoresis equipment may pose an electrical hazard if connections are made incorrectly. People with electronic or metallic implants should not enter within the fringe field of the NMR magnet.

RESULTS

Native polyacrylamide gel electrophoresis (PAGE) was used to characterize the native structure of the two peptides in both the absence and presence of Zn^{2+}. The designed peptide migrated significantly slower compared to the control peptide (see Supporting Information). The same pattern was observed in the absence and presence of Zn^{2+}.

Immobilized-metal affinity chromatography was used to test the ability of the two peptides to bind to Zn^{2+}. Designed and control peptides were loaded onto two separate iminodiacetic acid-agarose columns that had been charged with $ZnCl_2$ and then equilibrated with buffer. Columns were eluted first with equilibration buffer and 1.0 mL fractions were collected and monitored at 220 nm as shown in Figure 1. For the control

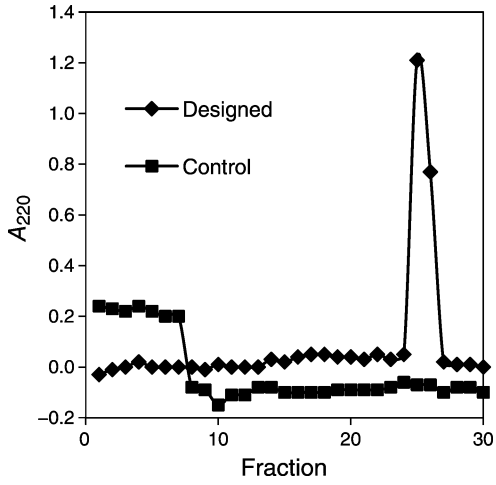

Figure 1. Immobilized-metal affinity chromatography elution profiles for designed and control peptides.

peptide, moderate A_{220} values were immediately observed, but tapered to baseline after 12 fractions, indicating that this peptide was not interacting with the column. For the designed peptide, A_{220} readings remained at baseline for the first 24 fractions. At this point, imidazole was added to the elution buffer. The A_{220} readings for fractions 25 and 26 were very large, but then returned to baseline at fraction 27, indicating that the peptide had been displaced from the column by the imidazole.

NMR spectroscopy was used to characterize the stoichiometry of zinc binding.[20] Peptide solutions were titrated with aliquots of a concentrated $ZnCl_2$ solution in increments of 0.25 equiv up to a 2-fold excess. The amide and aromatic resonance-

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containing region of the ^{1}H NMR spectra are compared in Figure 2. Progressive chemical shift changes are observed for the designed peptide as the quantity of Zn^{2+} is increased from 0 to 1.0 equiv. This is most readily apparent in the boxed regions between 7.1−7.2 and 8.3−8.4 ppm, which are the typical chemical shift regions for histidine side chain $^1H^{\delta 2}$ and $^1H^{\varepsilon 1}$ protons.[28] No further changes were observed in the designed peptide chemical shifts upon addition of Zn^{2+} up to 2.0 equiv. For the control peptide, small chemical shift changes were observed with the initial aliquot of $ZnCl_2$ but none thereafter.

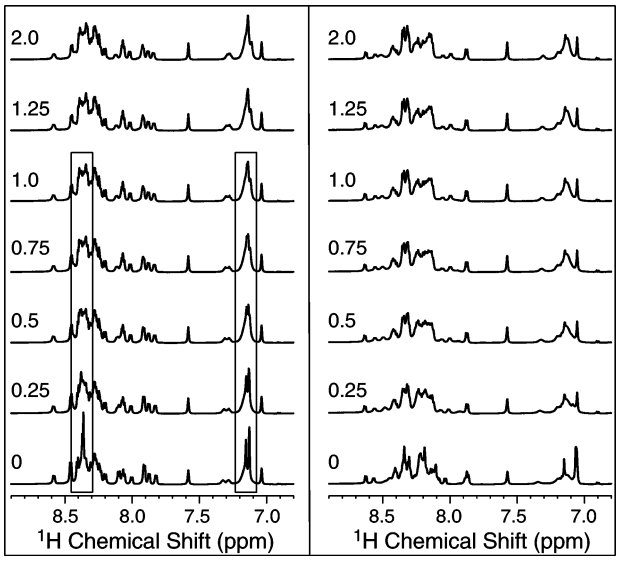

Figure 2. Amide and aromatic region of the 600 MHz ^{1}H NMR spectra of designed (left panel) and control (right panel) peptides with increasing amounts of Zn^{2+}. The ratio of Zn^{2+} to peptide is indicated on the left of each spectrum.

DISCUSSION

Compared to SDS-PAGE where protein mobility depends almost entirely on molecular mass, mobility on native PAGE depends on a combination of charge and hydrodynamic size. The mobility of the designed peptide was significantly reduced compared to the control peptide, suggesting that it may in fact be forming dimers or even higher-order aggregates. The observation that Zn^{2+} did not impact the mobility of the designed peptide indicated that formation of higher-order structures was independent of Zn^{2+} coordination.

The metal affinity column data indicated that the designed peptide had significantly greater affinity for Zn^{2+} than did the control peptide. The positive A_{220} values observed for the first seven fractions of the control peptide most likely reflected nonspecific interactions with the column matrix. The difference in baseline for the two peptides is not known. The elution profiles were collected by different groups of three students using separate columns and spectrophotometers, but the same buffer solutions were used in each experiment.

The ^{1}H NMR spectra indicated that the designed peptide not only coordinated Zn^{2+}, but also that it was more structured than the control peptide. For the designed peptide, chemical shift changes in the region of histidine side-chain resonances occurred up to the point where one equivalent of Zn^{2+} had been added, as would be expected for a single Zn^{2+} binding site. Zn^{2+}-dependent chemical shift changes were not observed in the control peptide. The greater chemical shift dispersion of the amide $^1H^N$ resonances for the designed peptide in the region of 7.7−8.7 ppm suggested that it was more structured compared to the control peptide. These structural differences could be explored in greater detail using various 2D ^{1}H NMR experiments.

Students entered the biophysical chemistry course with a solid understanding of protein structure and function obtained in their first-semester biochemistry course. The four-helix bundle design project allowed students to apply this basic knowledge to design a peptide sequence that would adopt a specific structure and possess a given biochemical function. The structure of the course with long, weekly class periods was conducive to a mix of computer simulations, literature discussions, and lab experiments. Moreover, the techniques used to test the designed peptide were complementary to what the students had experienced in their biochemistry laboratory course. Although the students were fascinated by working with Foldit, enthusiasm for the project in general, and for collecting experimental data in particular, increased upon actually seeing and holding the vials containing "their" peptides. A peptide in the lab is worth two on the screen.

ASSOCIATED CONTENT

Supporting Information

Instructor notes, companion homework assignment, post-lab questions, and student handout. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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