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MODIFYING HYDROXYAPATITE NUCLEATING PEPTIDES TO FORM NOVEL BORON CERAMICS

by
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14. ABSTRACT
Reported are efforts to modify the Hydroxyapatite nucleating peptide (HANP) isolated from Coryneabacterium matruchotti to nucleate boron as opposed to calcium. The literature reports that boron-binding proteins utilize a His and Ser complex to bind boron in their active sites. Using computer modeling, modifications were designed that changed the active site of HANP to include two His, two Ser or a Ser and a His. Four modified versions were proposed and each version was tested for their energy minimization. The computer models indicate the structures of all 4 were stable. The modified versions were cloned into E. coli and expressed. The expressed peptides were then tested for their ability to nucleate boron ceramics with no success. Two of the modified peptides were synthesized chemically and examined using circular dichromism(CD) and nuclear magnetic reasonance. The results suggest that the a-helical structure of HANP is lost with the modifications. The results from these experiments will be presented in detail and their implications discussed.

15. SUBJECT TERMS
HYDROXYAPATITE, BORON, BALLISTIC PROTECTION, PEPTIDES, CORYNEBACTERIUM, COMPUTER MODELING, CERAMICS, NANOTECHNOLOGY, PEPTIDE ANALYSIS, GENETIC ENGINEERING, CLONING, LIGHTWEIGHT, NUCLEATED MATERIALS

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PREFACE

This study was conducted at the Soldier Systems Center, U.S. Army Soldier and Biological Chemical Command, Natick MA 01760 between the period of 1 Oct 98 and 30 Sept 01 under Project Number AH52.

This report examines modifying existing ceramic peptides to nucleate boron type ceramics. It includes using computer modeling, genetic engineering, and peptide analytical techniques.

ACKNOWLEDGEMENTS

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MODIFYING HYDROXYAPATITE NUCLEATING PEPTIDES TO FORM NOVEL BORON CERAMICS

Introduction

Ballistic protection is an important research area for the Department of Defense. In a battle situation, soldiers are threatened with both fragment and projectiles (Maffeo et.al.). Current protection uses a soft component to prevent fragment penetration and a hard ceramic component to breakup faster projectiles. Though what is currently available offers excellent protection, it is quite heavy and adds a significant amount of weight to an already overloaded soldier.

Efforts to reduce the weight of ballistic protection by 30-50% while maintaining or improving current performance levels are on going. Since most of the weight is attributed to the ceramic component, much of the research has focused on reducing the plate weight. Of particular interest have been boron ceramics, which have been shown to be lighter than currently used ceramics while demonstrated desirable ballistic protection properties. The drawback to these ceramics is they are quite expensive and are quite difficult to produce involving toxic reagents, high temperatures and high pressures. Nanotechnology methods have been investigated to develop new procedures for producing boron ceramics.

Nanotechnology involves working at the atom scale to control formation and ultimately performance of a desired material. Until recently, nanostrategies have been from the field of chemistry, but biological approaches are developing. Organisms that form ceramic materials in nature have been studied and what has been learned provides the basis for synthetically derived ceramics using biologically nanostrategies.

The bacterium *Corynebacterium matruchotti* has the ability to nucleate Hydroxyapatite (HA). Small peptides exhibiting HA nucleation ability have been isolated from *C. matruchotti*, and characterizated. The

active site for these HA nucleating peptides (HANP), have been identified and a model has been proposed for HA nucleation (unpublished results). This study discusses efforts to develop a nanotechnology strategy for forming boron-based ceramics using modified versions of HANP. Though ultimately unsuccessful, a number of things were learned which could aid future efforts. These will be discussed within the text.

Materials and Methods

Determining Active Site Modification of Peptide

Proteins and peptides capable of binding boron utilize a Ser/His complex in their active sites (Katz et. al. 1995, Tsilikounas et. al.1993, Weber et. al. 1995). The HANP binds calcium using two Asp as determined by NMR analysis (unpublished work done at Bowling Green University). Models were proposed which change the Asp to His/Ser, Ser/His, His/His or Ser/Ser. Protein stability was determined by measuring the global minimal energy for each model. Models showing structural stability will be moved forward for cloning and nucleation studies.

Cloning the peptides for sequencing

Oligonucleatides coding for each of the four modified peptides and their compliments were synthesized (Great American Gene Company, Ramona, CA). Each set of oligonucleotides, were designed with a Kozak start codon, at the 5' end and a stop codon at the 3' end. A Not I site was included before the stop codon and a Hind III site after the codon to allow cloning with and without the His tag. An overhang complimentary to EcoR I was located at the 5' region and an overhang complimentary to Xba I at the 3'. Initially the insert was cloned into pPICZ B using the overhangs and the resulting clones sequenced.

Actual cloning involved denaturing the coding and complimentary strands at 95 °C for 5 min, annealed at 55°C for 10 min and ligating into pPICZ B (Invitrogen, San Diego, CA). The ligated DNA was ethanol precipitated, washed twice with 80% ethanol and electroporated into *E. coli* (TOPF') cells. The pulse settings were 250 kV, 200 Ω, 25μF. Cells were allowed to recover in 1 ml SOC buffer (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) at

37 °C for 1 h and colonies were isolated by spread plating onto LB agar (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, 2% agar) containing 30ug/ml Zeocin (Invitrogen). Individual colonies were minipreped and analyzed by gel electrophoresis. Colonies with inserts were sequenced.

Cloning for expression

The insert was digested with EcoR I/Not I and gel purified using the sephlaglas band prep kit (Amersham Pharmecia Biotech, Piscataway, NJ). The band was ligated into Pet 24 and transformed into *E.coli* (TOPF') as described above. Individual colonies were isolated, minipreped and sequenced to confirm the insert orientation. Plasmids containing inserts in the correct orientation were transformed into *E.coli* (HMS174(DE3)) (an expression cell line). Colonies were isolated and tested for expression.

Testing for Expression

Individual colonies were grown in 50 ml cultures of LB media containing kanamycin. The cultures were grown to an OD of 0.4-1 and induced for 3 hr with 1mM IPTG. Cells were collected by centrifugation, lyophilized and lysed in 6 M guanidine PT pH8.0. The lysate was clarified by centrifugation and the supernatant mixed with 1 ml of equilibrated NiNTA. The supernatant/Resin mix was allowed to incubate at 4°C overnight then poured into a column. The column was washed with 6 M guandine PT pH8.0, 6 M guandine PT pH6.3, 6M guandine PT pH 5.9 in succession and eluted in 6M Guandine PT pH4.3. Each wash was collected as a single fraction, dialyzed into 20% ethanol then lyophilized. The pellet was resuspended into 100 µl of 20% ethanol and analyzed on a 10-20% Tricine gel stained with silver or blotted onto a PVDF membrane for analysis via western hybridization using a anti-His antibody (Qiagen Inc., Valencia, CA).

Preparation of Samples for Circular Dichromism (CD) Analysis HANP and modified HANP versions 1 and 2 were synthesized (Cell Essentials, Boston, MA). Stock solutions for each peptide (1mM), were prepared by resuspending the peptide in MQ water. For analysis, 14 ul of the stock solution was added to a tube and

brought up to 300ul with buffer to a concentration of 0.0477 mM. For each peptide, three tubes were prepared, one as a control and the other with 5ul of either 30mM CaCl solution or 30 mM Boric acid solution. Samples were analyzed in Tris (5mM or 100mM) buffer, NaCl buffer, or MQ water alone.

Circular Dichromism (CD) analysis Each sample was placed into a quartz cuvette, with a 1mm path length. The sample was heated to 95 ° C then cooled back to RT with spectra being taken at 25, 35, 50, 65, 85 and 95° C. At each temperature, 5 spectra were taken and averaged. The data was plotted on excel.

Nuclear Magnetic Resonance (NMR) work The NMR data was obtained at Bowling Green University. NOESY and TOCSY were done for each sample.

Crystal Assay All assays were based upon the assay derived for HA nucleation. A metastable calcium or boron solution was prepared by dissolving 4.09g Sodium Chloride, 1.85g Sodium Bicarbonate, and 0.37g Potassium Chloride in 900ml of MQ water with either 0.276g Sodium Phosphate (dibasic) for calcium or 0.165g Sodium Nitrate for boron. The chemicals were completely dissolved into 900ml of water and the pH was adjusted to 6.6 using CO_2 (Dry ice in water generated the CO_2). Just before being used, 100ml of a Calcium buffer (0.296g Calcium Chloride and 0.2g Thymol filtered through a 0.22uM filter) or 100 ml of Boron buffer (either 0.77g Borax or 0.12g Boric acid with 0.2g thymol filtered through a 0.22 um filter) was added to the solution. The pH was adjusted to 7.2 using Nitrogen gas and aliquoted in 1.5 ml amounts to epindorf tubes containing 30 ul of the test protein. The mixture was allowed to incubate for 24-48 hr and examined for crystals via light microscopy or TEM.

Results

Cloning the Modified Versions

Oligonucleotides coding for the each modified peptide (Fig 1) were synthesized and successfully cloned into E.coli. For each set of clones, 3-5 colonies containing and insert were isolated and sequenced. Sequencing results indicated not all of the inserts were complete with about 50% showing deletions (Fig 2).

Mod 1

Met-Gln-Phe-Ile-Thr-*His*-Leu-Ile-Lys-Lys-Ala-Val-*Ser*-Phe-Phe-Lys-Gly-Leu-Phe-Gly-Asn-Lys-Gln

5- AAT-TCA-AGA-TGC-AGT-TCA-TCA-CTC-ACC-TTA-TCA-AAA-AAG-CTG-TTA-GCT-TCT-TCA-AAG-GTC-TTT-TCG-GTA-ACA-AAC-AGA-TGG-CGG-CCG-CCT-AAG-CTT -3'

5-CTA-GAA-GCT-TAG-GCG-GCC-GCC-ATC-TGT-TTG-TTA-CCG-AAA-AGA-CCT-TTG-AAG-AAG-CTA-ACA-GCT-TTT-TTG-ATA-AGG-TGA-GTG-ATG-AAC-TGC-ATC-TTG-3' Compliment

Mod 2

Met-Gln-Phe-Ile-Thr-*Ser*-Leu-Ile-Lys-Lys-Ala-Val-*His*-Phe-Phe-Lys-Gly-Leu-Phe-Gly-Asn-Lys-Gln

5-AAT-TCA-AGA-TGC-AGT-TCA-TCA-CTA-GCC-TTA-TCA-AAA-AAG-CTG-TTC-ACT-TCT-TCA-AAG-GTC-TTT-TCG-GTA-ACA-AAC-AGA-TGG-CGG-CCG-CCT-AAG-CTT-3'

5-CTA-GAA-GCT-TAG-GCG-GCC-GCC-ATC-TGT-TTG-TTA-CCG-AAA-AGA-CCT-TTG-AAG-AAG-TGA-ACA-GCT-TTT-TTG-ATA-AGG-CTA-GTG-ATG-AAC-TGC-ATC-TTG-3' Compliment

Mod 3

Met-Gln-Phe-Ile-Thr-*His*-Leu-Lys-Lys-Ala-Val-*His*-Phe-Phe-Lys-Gly-Leu-Phe-Gly-Asn-Lys-Gln

5-AAT-TCA-AGA-TGC-AGT-TCA-TCA-CTC-ACC-TTA-TCA-AAA-AAG-CTG-TTC-ACT-TCT-TCA-AAG-GTC-TTT-TCG-GTA-ACA-AAC-AGA-TGG-CGG-CCG-CCT-AAG-CTT-3'

5-CTA-GAA-GCT-TAG-GCG-GCC-GCC-ATC-TGT-TTG-TTA-CCG-AAA-AGA-CCT-TTG-AAG-AAG-TGA-ACA-GCT-TTT-TTG-ATA-AGG-TGA-GTG-ATG-AAC-TGC-ATC-TTG-3' Compliment

Mod 4

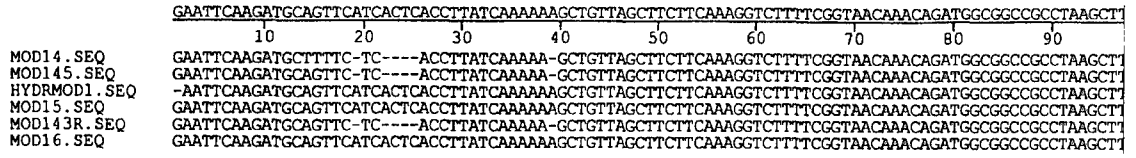
Met-Gln-Phe-Ile-Thr-*Ser*-Leu-Lys-Lys-Ala-Val-*Ser*-Phe-Phe-Lys-Gly-Leu-Phe-Gly-Asn-Lys-Gln

5-AAT-TCA-AGA-TGC-AGT-TCA-TCA-CTA-GCC-TTA-TCA-AAA-AAG-CTG-TTA-GCT-TCT-TCA-AAG-GTC-TTT-TCG-GTA-ACA-AAC-AGA-TGG-CGG-CCG-CCT-AAG-CTT-3'

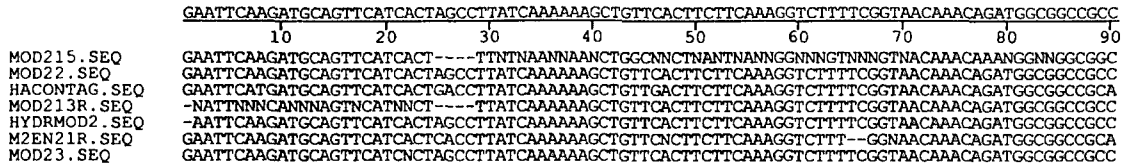
5-CTA-GAA-GCT-TAG-GCG-GCC-GCC-ATC-TGT-TTG-TTA-CCG-AAA-AGA-CCT-TTG-AAG-AAG-CTA-ACA-GCT-TTT-TTG-ATA-AGG-CTA-GTG-ATG-AAC-TGC-ATC-TTG-3' Compliment

Figure 1. **Sequences for Boron Ceramic Proteins**

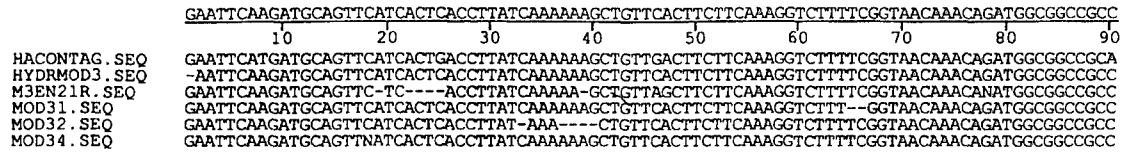
Modified version 1



Modified version 2



Modified version 3



Modified version 4

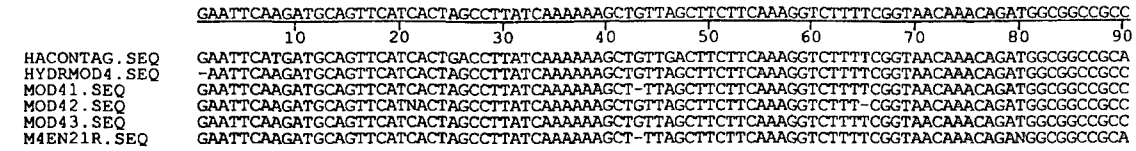


Figure 2. **Sequenced Data Showing Incomplete Sequences Due to Deletions.**

Modified version 2-4 were compared to the unmodified version labeled HACONTAG

Clones with the correct inserts were used for cloning into expression systems. No deletions were detected in the expression clones.

Expression for the Modified Versions

Four isolates per set of clones were tested for expression. Anti HA antibody would not bind to the modified versions as determined by a dot blot. Anti His antibody bound to the peptides but showed a significant amount of non-specific binding in the cell lysates. Each lysate was run over a Ni-NTA column dramatically reducing the background. The target protein eluted with the pH 4.3 wash and was reasonably pure as determined by silver staining. Though yields were not determined, intensity of the bands suggested modification peptide 1 had expressed in a higher amount than the other peptides.

CD work of Synthetic Version

CD analysis of synthetically produced peptides 1 and 2 revealed a loss of secondary structure. Initial measurements followed the Bowling Greens University protocol and were made in an un-buffered solution (Fig 3). Under these conditions, the HANP control did not duplicate Bowling Greens spectra and showed a loss of alpha helix and with a gain of random coil. The HANP control was reanalyzed using 5mM tris buffer and regained the previously reported curve (Fig 4). The CD spectra for the modified peptides in either un-buffered or buffered solutions remained the same. The resulting curve suggests very little alpha helix with a high degree of random coil. The addition of calcium or boric acid to any of the peptides showed little if any discernible difference on the CD spectra.

NMR Results:

NMR spectra were taken at Bowling Green University and analyzed using the Felix 95 program. As with the CD, the results of the unmodified HANP resembled previously reported results suggesting HANP has a very high degree of α–helix. The NMR spectra taken for modified peptide 2 were quite different from those obtained for HANP (Fig 5 and 6) and suggested the α–helix had been replaced by random coil. Attempts to obtain NMR spectra of modified version 2 in the presence of boron were unsuccessful.

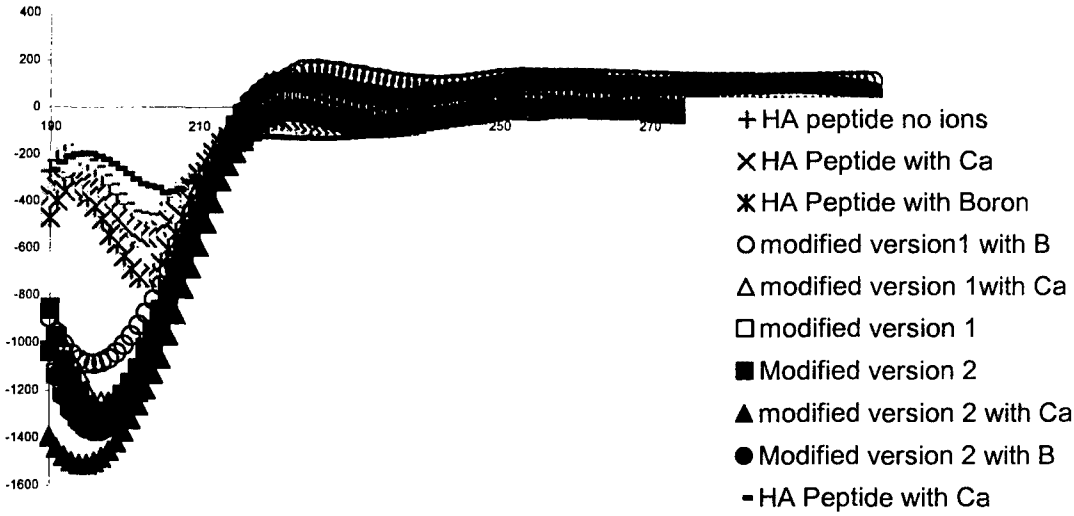


Figure 3. **Comparison of the CD Results for HA and Modified Version with No Buffer.** The HA peptide with Calcium was run twice .

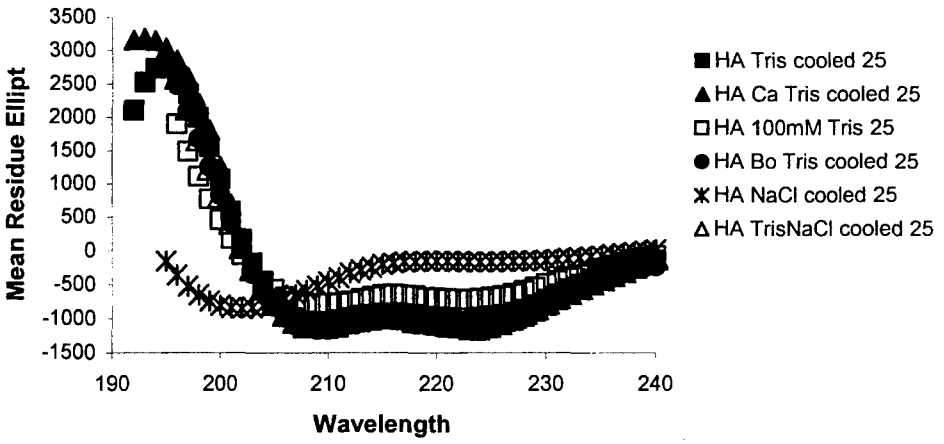


Figure 4. **Comparison of CD Spectra in the Presence of a Buffer.**

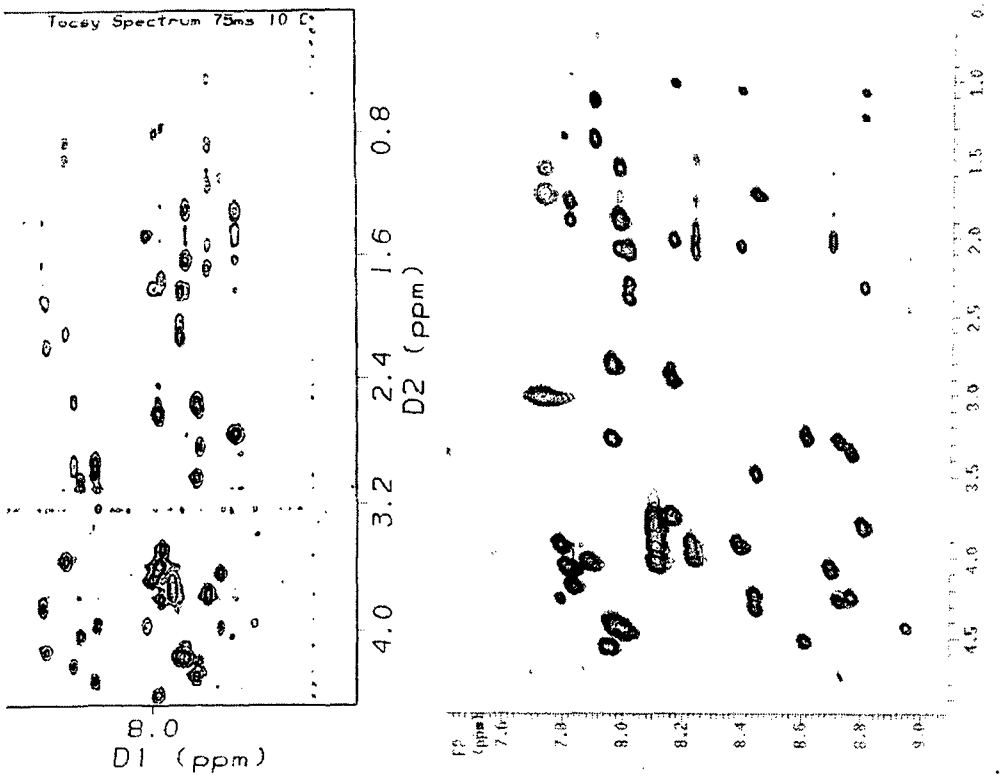


Fig 5 and 6. **The Toesy of HA (left) and Modified Version 2** (right). Though the scale is slightly different, it is obvious there are some significant peak shifts. These peptides do not have similar structures.

The addition of the boric acid caused the peptide to precipitate out of solution. No NMR results could be obtained for Modified peptide 1.

Discussion

All 4 modified versions and the HANP control were successfully expressed in *E. coli* as determined by western hybridization. The deletions found in some of the initially cloned inserts were unexpected. The genes were created using synthesized oligonucleotides and its been speculated that one of the oligonucleotides may have a form a natural loop. This loop would have resulted in the polymerase "skipping" over some bases during replication resulting in a deletion. It might be possible to "breakup" this loop by increasing the annealing temperature. Since some of the clones were correct this was not tried.

The loss of the secondary structure was not predicted. Computer models indicated the modifications would keep the alpha helical nature of HANP, but as observed by CD and NMR, this was not correct. The failure of the models reflects the lack of understanding of protein folding or at least the factors that drive it. The effects of the structural loss are reflected in the solubility problems.

The modified peptide #2 precipitated out of solution when boric acid was added. The protein sequence for HANP is quite hydrophobic and remains soluble by folding the hydrophobic regions in leaving the hydrophilic portions on the outside. The loss of the alpha helical structure left the hydrophobic regions exposed, thereby decreasing solubility. Though the modified peptides were slightly soluble in the NMR test solvent, they were easily salted out by the boric acid. This would be a problem in forming boron type crystals. It would be necessary to further modify the sequence to reestablish the alpha helical nature and decrease the hydrophobicity of the peptide. Substituting hydrophobic amino acids like isoleucine, leucine and valine with hydrophilic, helix inducing amino acids like alanine or glycine should increase solubility and promote alpha helix formation.

The structural loss may also explain why the anti HA antibody would not bind to the modified versions. Antibodies bind to specific peptide sequences in a specific configuration. The point of the experiment changed the sequence and obviously the structure. The antibody could not handle changing both.

Crystal formation is unlikely given the structural changes. The active site for the unmodified HANP forms HA because of the peptide alpha-helical nature. The two Asp residues are in close proximity as the peptide turns allowing binding of the Ca ions. The Lys residues, which bind the phophate groups, are in position because of the helical nature of the protein. The loss of the helix folds the lysine away from each other preventing phosphate binding. Because of the solubility problems, NMR could not be used to support this hypothesis, but crystal formation experiments suggested the peptides were not functioning. A potential test to show support for the view would be to disrupt the helix of the native peptide while keeping the active sites intact. This should deactivate the peptide if the theory is correct. Due to time this was not done.

Conclusions

Though ultimately unsuccessful in creating a boron nucleating peptide, there are some positives. The cloning work was very successful. Each peptide was successfully cloned and expressed. Though the original computer models did not predict the structural changes, we have gained a better understanding of protein modeling. This understanding can be used to obtain better programs to predict protein secondary structure.

Using proteins to nucleate ceramics is still considered a viable approach to nucleating desired ceramics. There are two potential directions this research could go in. It maybe possible to further modify the peptides to increase solubility and return the structure to an alpha helix. This may or may not allow the peptide to nucleate boron. Certainly boron binding should be achieved. The other approach would be to

use a random pool of peptides and search for peptides with boron nucleating ability. Phage display and mRNA display proteins are two methods commonly used for "mining" peptides pools for specific traits or characteristic. Either approach could be used to identify boron binding/nucleating peptides.

This document reports research undertaken at the U.S. Army Soldier and Biological Chemical Command, Soldier Systems Center, Natick, MA, and has been assigned No. NATICK/TR-03/030 in a series of reports approved for publication.

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