



Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1501–1505

BIOORGANIC &
MEDICINAL
CHEMISTRY
LETTERS

Methodology for Optimizing Functional Miniature Proteins Based on Avian Pancreatic Polypeptide Using Phage Display

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Received 21 November 2000; accepted 8 February 2001

Abstract—Synthetic genes for avian pancreatic polypeptide (aPP) and for the miniature DNA binding protein PPBR4 were cloned and expressed on the surface of M13 bacteriophage. We anticipate that these constructs will have utility optimizing the properties of miniature proteins based on aPP that result from our previously described protein grafting procedure. © 2001 Elsevier Science Ltd. All rights reserved.

Many proteins recognize nucleic acids, other proteins or macromolecular assemblies using a partially exposed α -helix. Within the context of a native protein fold, such α -helices are usually stabilized by tertiary interactions with residues that may be distant in primary sequence from both the α -helix and from each other. With notable exceptions,¹ removal of these tertiary interactions destabilizes the α -helix and results in molecules that neither fold nor function in macromolecular recognition.² The ability to recapitulate or perhaps even improve upon the recognition properties of an α -helix within the context of a small molecule should find utility in the design of synthetic mimetics or inhibitors of protein function³ and as new tools for proteomics research.

Two fundamentally different approaches have been taken to bestow α -helical structure on otherwise unstructured peptide sequences. One approach makes use of modified amino acids or surrogates that favor helix initiation⁴ or helix propagation.^{5–7} Perhaps the greatest success has been realized by joining the *i* and *i*+7 positions of a peptide with a long-range disulfide bond. Peptides containing this artificial constraint can, in certain cases, retain helical structure at temperatures as high as 60 °C.⁸ A second approach^{3,9} useful for proteins containing α -helix or β -sheet structure, is to pare the extensive tertiary structure surrounding a given recognition sequence to generate the smallest possible functional molecule. This strategy has generated minimized

versions of the Z domain of protein A and atrial natriuretic peptide, which contain 59 and 28 amino acids, respectively. The minimized proteins, which contain 33 and 15 amino acids, respectively, were well-folded and displayed high biological activity.^{10,11} Despite this success, it is difficult to envision a simple and general application of this strategy in the large number cases where the α -helical epitope is stabilized by residues scattered throughout the primary sequence.

In light of this limitation, we explored a different and potentially more flexible approach to protein minimization called protein grafting (Fig. 1). Schematically, protein grafting involves removing residues required for molecular recognition from their native α -helical context and grafting them on the scaffold provided by the very small yet stable protein avian pancreatic polypeptide (aPP). aPP is a 36 amino acid peptide whose structure contains an α -helix (residues 14–32) and a type II polyproline helix (residues 1–8) joined by a type I β -turn. aPP exhibits a cooperative thermal melt with a T_m of 60 °C (though this value may represent data for an aPP multimer¹²). The stability of aPP derives primarily from hydrophobic interactions between interior residues on the two helices,¹³ predicting that the exterior surface could be used for recognition. Because of its small size and stability, aPP is an excellent scaffold for protein grafting of α -helical recognition epitopes.²

In contrast to the case for traditional protein minimization, it is easy to envision how protein grafting could stabilize almost any α -helical epitope, since the necessary stabilizing interactions can theoretically be provided by

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the aPP scaffold irrespective of the tertiary complexity of the natural protein. For this reason our strategy could in theory be generalized to minimize any protein that recognizes a receptor using up to five turns of an α -helix.

Recently we demonstrated that protein grafting could be used successfully to minimize the basic region of the bZIP protein GCN4.² Grafting of the GCN4 residues responsible for DNA recognition¹⁴ onto the exterior surface of the aPP α -helix yielded the 42 amino acid peptide PPBR4.² PPBR4 recognized specific DNA well at 4 °C, forming a complex with an equilibrium dissociation constant (K_d) of 2.3 nM. However, PPBR4, like the isolated GCN4 basic region,¹⁵ was itself unstructured and failed to bind DNA at ambient temperatures. Indeed, our experience with multiple systems suggests that molecules designed solely by protein grafting often fold poorly and can be improved significantly through structural imprinting by functional selection.¹⁶

In the second step of protein grafting, we use phage display¹⁷ to improve the properties of our miniature proteins. Libraries of miniature protein variants designed to improve either folding or binding (or both) are displayed on phage and undergo a functional selection on the basis of their affinity for a target molecule (Fig. 1).

Herein we report: (i) a synthetic gene allowing for the expression of aPP (which has previously been accessed by purification from avian pancreas¹⁸ or peptide synthesis) and PPBR4 in *Escherichia coli*; (ii) phagemids that allow rapid cloning of aPP-based miniature proteins; (iii) experiments demonstrating that phage displaying aPP are produced from cells harboring the appropriate

phagemid; and (iv) that miniature proteins with improved binding properties can be selected on the basis of function. These experiments represent important first steps towards optimizing a range of miniature proteins that bind DNA, RNA, and protein targets with high affinity and selectivity.

Results and Discussion

Vector construction

The phage display vector pJC20 was derived from the monovalent phage display vector pCANTAB5E (Pharmacia). pJC20 was prepared by inserting a synthetic gene encoding aPP between the unique Sfi I and Not I restriction sites found in pCANTAB5E. The synthetic aPP gene contained codons for optimal protein expression in *E. coli* and four restriction sites (Xma I, Age I, Bgl II, and Pst I) absent in pCANTAB5E. These restriction sites allow for the efficient construction of genes encoding a variety of discrete miniature proteins as well as for the introduction of genetic diversity (Fig. 2). The vector pJC21 was prepared by inserting a synthetic gene encoding residues 18–42 of PPBR4 between the unique Bgl II and Not I sites in pJC20. The identities of pJC20 and pJC21 were confirmed by automated DNA sequencing.

Avian pancreatic polypeptide is expressed on the surface of M13 phage

As a first step towards displaying miniature proteins on the surface of phage, we sought to verify that aPP was expressed from our synthetic gene, which is under the control of a lac promoter. To this end, TG-1 *E. coli* harboring pJC20 were induced with isopropylthiogalactoside (IPTG), lysed, and the cell lysates probed with a rabbit anti-aPP antibody (Peninsula Laboratories #RGG-7194). This experiment showed clear evidence for IPTG-inducible expression of aPP fused to the minor capsid protein III of M13 bacteriophage (Fig. 3a). To investigate whether this fusion protein was assembled into viable phage particles, we purified the phage, resolved the phage proteins using SDS PAGE, and probed with the same rabbit anti-aPP antibody. The Western blot (Fig. 3b) clearly shows that the fusion protein containing aPP and protein III is incorporated into fully assembled M13 phage particles. No signal was observed when phage proteins produced by cells harboring pJC21 were probed with the rabbit anti-aPP antibody.

Miniature proteins displayed on phage can be functionally selected

The first example of a miniature protein generated by protein grafting is PPBR4.² As a first step towards optimizing the function of PPBR4, we confirmed that phage displaying PPBR4 could be selected over phage bearing aPP when the two were sorted on the basis of specific DNA-binding.¹⁹ Phage displaying either PPBR4 or its progenitor aPP were panned against magnetic

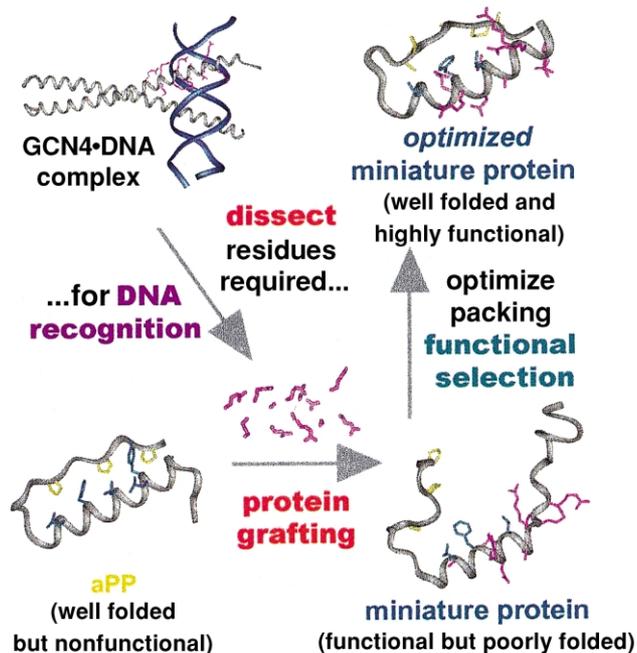


Figure 1. Schematic representation of the protein grafting procedure for miniature protein design and optimization.

beads coated with a 24 bp duplex oligonucleotide containing the 5 bp sequence recognized by PPBR4, half site CRE (hsCRE, ATGAC). The DNA was attached to streptavidin coated beads through a 3' biotin TEG (triethyleneglycol) linker (Glen Research). Panning was performed essentially as described by Choo and Klug.¹⁹ Wash conditions were optimized to maximize differential retention of phage displaying PPBR4 and aPP. In phosphate buffered saline (PBS) supplemented with 0.1% NP-40, 0.4 mg/mL BSA and 2.5 µg/mL poly dIdC, the percent retention of PPBR4 phage on hsCRE-coated beads was 10 times greater than that of aPP

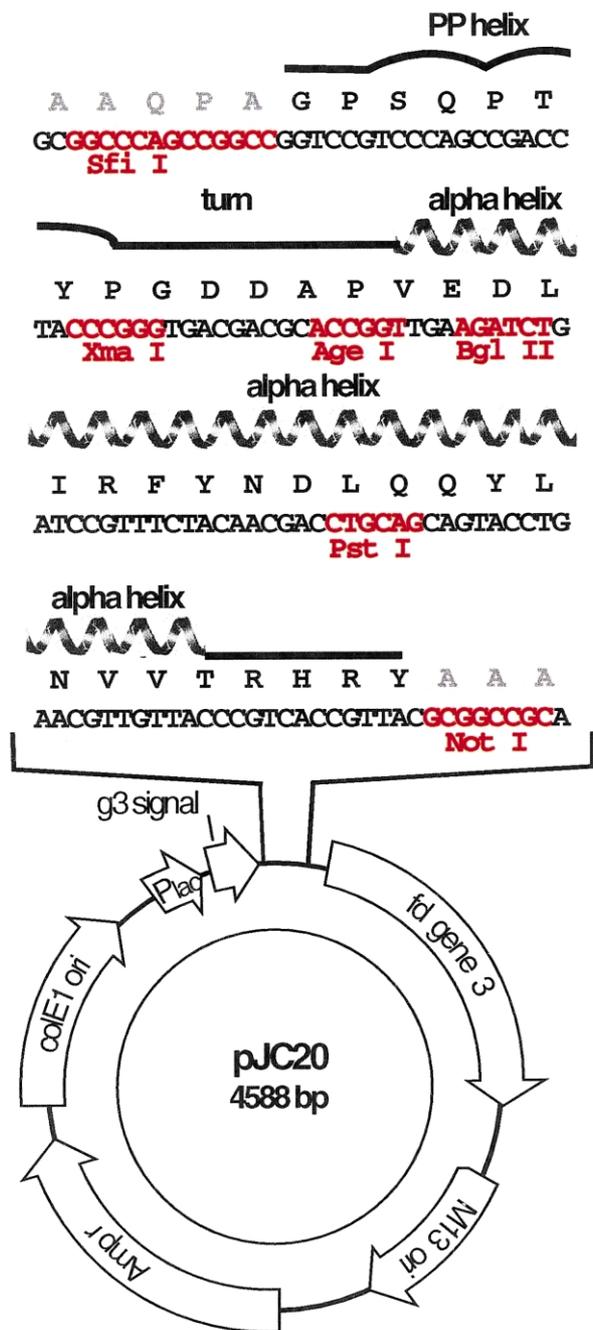


Figure 2. A map of pJC20. This vector contains the aPP coding sequence inserted between the unique Sfi I and Not I sites in PCAN-TAB5E.

phage (Fig. 4). This result suggests that miniature proteins generated by protein grafting may be functionally selected on M13 phage.

Experimental

Construction of synthetic genes

A synthetic gene for aPP was constructed using codons chosen to optimize expression in *E. coli* and incorporated four unique restriction sites to facilitate cassette mutagenesis. The 142 bp duplex insert was generated by use of mutually primed synthesis and the oligonucleotides APP.TS (CTATGCGGCCAGCCGGCCG GTCCGTCCCAGCCGACCTACCCGGGTGACGACGCACCGGTTGAAGATCTGATCCGTTTCTACACGACCTGCAGCAGTACCTGAACGTTGTTACCCGTCACCGTTACGCGGCCGAGGTGCG) and APP.BS (CTATGCGGCCAGCCGGCCGGTCCG-TCCCAGCCGACCTACCCGGGTGACGACGCA-CCGGTTGAAGATCTGATCCGTTTCTACAACG) which overlap at 19 bp. Our reaction mixture (20 µL) contained 8 pmol APP.TS, 8 pmol APP.BS, 1X ThermoPol buffer (New England Biolabs), 2 µg BSA, 1 mM dNTPs, 25 µCi [³²P] ATP, 5 mM MgSO₄, and 2 µL Vent(exo-) DNA polymerase and was incubated at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 1 min.

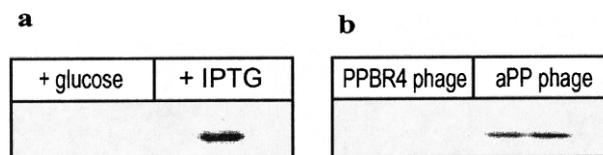


Figure 3. The aPP synthetic gene is expressed and incorporated into M13 phage particles. (a) Western blot of lysates of TG-1 *E. coli* harboring pJC20 grown in the presence of either the catabolite repressor glucose or IPTG. aPP expression is observed only upon addition of IPTG. (b) Western blot of whole phage produced by TG-1 *E. coli* harboring pJC20 (aPP phage) or pJC21 (PPBR4 phage).

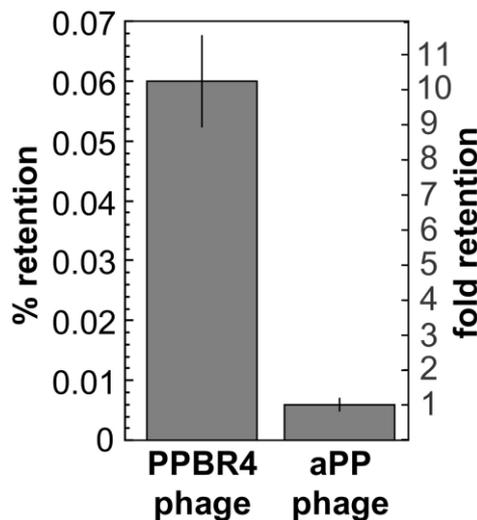


Figure 4. Percent retention of PPBR4 phage and aPP phage on magnetic beads coated with hsCRE. Error bars represent the standard error of three trials.

The major reaction product was purified from a denaturing (8 M urea) 10% acrylamide (29:1 acrylamide/bisacrylamide) gel and amplified by PCR in a 100 μ L volume containing 1500 pmol of the primers CTATGCGGCCAGCCGCGCCGG and CGCACCTGCGGCCGCGTAACG, 10 μ L template, 0.25 mM dNTPs, 5 mM MgSO₄, 1X ThermoPol buffer (New England Biolabs), and 2 μ L Vent(exo-) (New England Biolabs). The PCR reaction was subjected to 30 cycles of denaturation (94 °C for 30 s), annealing (60 °C, 30 s) and extension (72 °C, 1 min). The insert was digested with Sfi I at 50 °C in New England Biolabs buffer 2 for 4 h. This buffer was then supplemented with NaCl to a final concentration of 100 mM and with Tris–HCl to a final concentration of 50 mM before digestion with Not I for 4 h at 37 °C. The resulting insert was ligated into the vector pCANTAB5E (Pharmacia) in a reaction containing 800 U T4 DNA ligase (New England Biolabs), 50 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 25 μ g/mL BSA, 1 mM ATP, 250 ng pCANTAB5E at 16 °C for 1.5 h. The ligation products were transformed by electroporation into TG1 *E. coli* and the resulting plasmid named pJC20. A synthetic gene for PPBR4 was generated by replacing 57 bp at the 3' end of the aPP synthetic gene (in pJC20) with the sequence encoding the C-terminal 25 amino acids of PPBR4. The oligonucleotides PPBR4TS (GATCTGAAGCGCTTT CGTAACACCCTGGCTGCGCGCCGTTCCCGTGC ACGTAAAGCTGCACGTGCTGCAGCTGGTGGTT GCGC) and PPBR4BS(CGCACCTGCGGCCGCGCAACCACCAGCTGCAGCACGTGCAGCTTTACG TGCACGGGAACGGCGCGCAGCCAGGGTGTTA CGAAAGCGCTTCAGATCTTCAACC) were annealed and phosphorylated on the 5' end to form the PPBR4 insert. The PPBR4 insert was ligated into pJC20 that had been previously digested with Bgl II and Not I and dephosphorylated with calf intestinal phosphatase (New England Biolabs). The ligation reaction mixture contained 800 U T4 DNA ligase in 50 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 25 μ g/mL BSA, 1 mM ATP, 90 ng digested pCANTAB-5E, and 8 ng annealed insert. After reaction, the ligation mixture was transformed into electro-competent TG1 *E. coli*. The plasmid was named pJC21. The sequences of all final constructs were confirmed by automated sequencing.

Phage production

A 10 mL volume of 2xYT containing 100 μ g/mL ampicillin and 2% glucose was inoculated with a 500 μ L overnight culture of TG-1 *E. coli* containing the plasmids pJC20 or pJC21 and shaken at 37 °C to an OD₆₀₀ of 0.8. 4 \times 10¹⁰ pfu of M13 KO7 helper phage were added and shaking continued for an additional 1 h. Cells were pelleted for 15 min at 5000g and resuspended in an equal volume of 2xYT containing 100 μ g/mL ampicillin and 50 μ g/mL kanamycin and grown for 10 h with shaking. Cells were pelleted by centrifugation at 5000g for 20 min and the phage supernatant filtered through a 0.45 μ m filter before precipitation with PEG/NaCl (20% w/v PEG 8000 (Sigma), 2.5 M NaCl in ddH₂O) on ice for 45 min. Phage were pelleted at 13,000g for 30 min at 4 °C and resuspended in binding buffer.

Western blots of TG1 cell lysates

TG1 cells containing pJC20 were grown for 1 h at 30 °C in 2xYT containing ampicillin at 100 μ g/mL and 2% w/v glucose. Cells were pelleted by centrifugation at 5000g and resuspended in an equal volume of 2xYT containing 100 μ g/mL ampicillin and 1 mM IPTG, grown for 3 h at 30 °C and then lysed by boiling in SDS sample buffer.²⁰ Aliquots were loaded onto a Pharmacia Phast HOMO 20 gel and electrophoresed at 95 V·h until the solvent front ran off the gel. Proteins in the gel were transferred at 65 °C for 1 h to an Immobilon-P membrane. The membrane was blocked for 30 min with TBST (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20) containing 0.5% BSA and then incubated with a 1:10,000 dilution of rabbit anti-aPP (Peninsula Laboratories RGG-7194) provided at 4 mg/mL. The membrane was then washed three times (5 min per wash) with TBST, and then incubated with TBST containing a goat anti-rabbit alkaline phosphatase conjugate (Santa Cruz sc-2007) at a 1:1000 dilution. After three 5 min washes with TBST and a single wash with TBS (TBST lacking Tween-20), the membrane was stained with VISTRA ECF (Pharmacia) and visualized at 405 nm on a STORM 850 Phosphorimager (Molecular Dynamics).

Western blots of proteins isolated from phage particles

Phage (10 mL) were produced and precipitated with PEG/NaCl as described above. The precipitated phage were then resuspended in 1 mL ddH₂O, precipitated with 200 μ L PEG/NaCl, resuspended in 100 μ L ddH₂O and heated to 95 °C in SDS sample buffer for 10 min. The phage proteins were then applied to a 10% SDS gel (29:1 acrylamide/bisacrylamide) and subjected to electrophoresis at 20 mA in Tris–glycine electrophoresis buffer until the solvent front just ran off the gel. The separated proteins were transferred to an Immobilon-P membrane (Millipore) at 20 V for 4 h using a TE62 unit (Pharmacia) containing Towbin buffer (20% MeOH, 25 mM Tris–HCl (pH 8), 192 mM glycine, 0.1% SDS (w/v)) at 4 °C. After blocking with 5% nonfat milk (Carnation) in TBST for 16 h and washing twice (5 min per wash) with TBST, the membrane was probed for 30 min with anti-aPP in TBST supplemented with 2.5% nonfat milk. The membrane was washed three times (5 min per wash) with TBST, then exposed for 15 min to a goat anti-rabbit antibody–alkaline phosphatase conjugate (Santa Cruz sc-2007) at a 1:5000 dilution in TBST supplemented with 2.5% nonfat milk. After washing three times (5 min per wash) with TBST and twice (5 min per wash) with TBS the membrane was stained with Vistra ECF (Pharmacia) and visualized at 405 nm on a Storm 850 phosphorimager (Molecular Dynamics).

Panning

Streptavidin-coated M-280 magnetic beads (0.5 mg) (Dynal) were washed six times with 50 μ L of 2 \times B + W buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2.0 M NaCl). Each wash step was performed for 2 min. The beads were blocked upon incubation in 50 μ L of 1 \times B + W containing 6% nonfat milk for 14 h. The

beads were then washed five times with 50 μ L 1 \times B+W and incubated for 12 min in 50 μ L 1 \times B+W containing approximately 1 μ M duplex hsCRE₂₄² carrying a 3' biotin label on one strand. This procedure loaded approximately 75 pmol DNA per mg of beads. The beads were then washed five times with 50 μ L phage binding buffer (phosphate buffered saline supplemented with 0.4 mg/mL BSA, 0.1% NP-40 and 2.5 μ g of poly dIdC). 10¹⁰ phage in a volume of 0.4 mL were added to the beads at 4 °C and incubated with rotation on a LABQUAKE shaker rotisserie for 2 h. Beads were washed five times for 5 min at 4 °C with wash buffer (phage binding buffer lacking poly dIdC). Bound phage were eluted at 25 °C for 2 h by addition of wash buffer containing 4 M NaCl. 200 μ L of the elution and 200 μ L of phage not subject to panning were each used to infect 7 mL of log phase TG-1 *E. coli*. After 1 h, serial dilutions of infected cells were plated on SOBAG (SOB media supplemented with 2% w/v glucose and ampicillin to 100 μ g/mL) and grown for 12 h at 30 °C. Values of percent retention were calculated from the equation: percent retention = (output titer/input titer) \times 100.

Summary

We have shown that aPP can be displayed on the surface of M13 phage. We have also shown that miniature proteins based on aPP can be functionally selected when displayed on the phage surface. These experiments suggest that selection by phage display is a viable strategy for optimizing the properties of miniature proteins such as PPBR4. Indeed, we have recently constructed several libraries of miniature proteins on phage and panned them successfully for improved properties. The results of these experiments will be reported in due course.

Acknowledgements

J.W.C. thanks the Fulbright Commission and the Arthur Wayland Dox Foundation for predoctoral fellowships and Stacey E. Rutledge for assistance with phage Western blotting. R.M.G. and M.A.F. thank the

Anna Fuller Fund and the NIH, respectively, for post-doctoral fellowships.

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