

Highly Specific DNA Recognition by a Designed Miniature Protein

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Many DNA-binding proteins employ a short α -helix as their primary DNA recognition element.^{1–5} When removed from their natural contexts, however, most α -helices neither fold nor bind DNA with high affinity.^{6,7} Here we describe a strategy for the design of miniature proteins that present a solvent-exposed α -helix able to recognize DNA with high affinity and specificity. This strategy involves dissecting those α -helical residues required for DNA recognition from their native protein context and grafting them on a stable, miniature protein core (Figure 1). This strategy was used to design a 42-amino acid protein displaying the DNA-contact residues of a single basic region-leucine zipper (bZIP) protein. This miniature protein bound DNA in the low nanomolar concentration range under physiological solution conditions and exhibited greater sequence specificity than a natural bZIP protein. This strategy may represent a general approach to the design of small, folded proteins that recognize nucleic acid and protein targets with high affinity and specificity.

Our design process began with avian pancreatic polypeptide (aPP), a small, well-folded protein of known structure consisting of a single α -helix stabilized by hydrophobic interaction with a type II polyproline helix (Figure 1).^{8–10} Since formation of the aPP core requires residues on only one face of the aPP α -helix (shown in blue), the opposite, solvent-exposed face of the α -helix is available for recognition of other macromolecular targets. By grafting various combinations of those thirteen residues (shown in pink) used by GCN4 to recognize the CRE half site (ATGAC, hsCRE)^{1,2} on the solvent-exposed α -helical face of aPP, we generated a series of polyproline helix-basic region (PPBR^{SR}) molecules containing most or all of the DNA-contact residues of GCN4 and most or all of the folding residues of aPP (Figure 2).

This procedure generated three positions of conflict, where essential DNA-contact and aPP-folding residues occupied a single position on the helix (Figure 2). No significant DNA binding was detected with peptides PPBR0^{SR}, PPBR10^{SR}, and PPBR11^{SR}, which lacked one or more of these DNA-contact residues. High-affinity DNA binding was observed with a peptide that contained these three residues: The equilibrium dissociation constant (K_d) of the PPBR2^{SR}·hsCRE complex was 5 nM under conditions of physiological ionic strength (Figure 3). DNA affinity was enhanced further by selective alanine substitutions that increased the overall α -helical propensity of the peptide, producing the

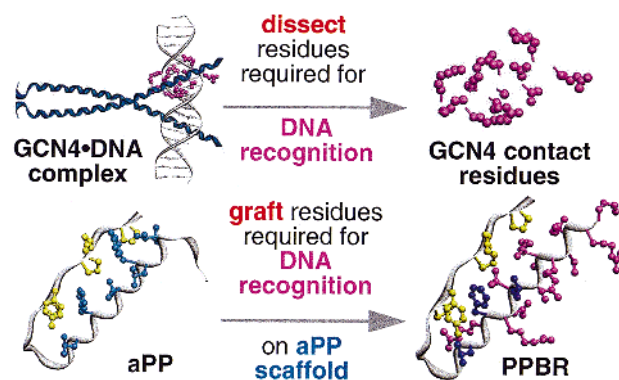


Figure 1. Protein grafting strategy for the design of miniature DNA-binding proteins.

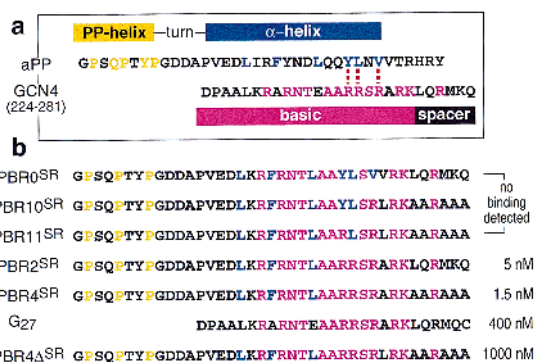


Figure 2. (A) Alignment of the aPP and the GCN4 basic-spacer segment sequences used to guide protein design. Essential DNA-contact residues within GCN4 are in pink; essential folding residues within aPP are in yellow or blue. Conflict positions are indicated by a dashed line. (B) Peptides used and their affinities for hsCRE₂₄. Equilibrium dissociation constants of stable PPBR^{SR}·hsCRE complexes are listed at right. All peptides except G₅₆²⁸ and G₂₇ contained GGC sequences at their carboxyl termini;²⁹ G₂₇ contained a single cysteine. The carboxy-terminal cysteine was alkylated with bromoacetamide to study protein monomers (PPBR^{SR}, G₂₇) or oxidized to study disulfide-linked dimers (PPBR^{SS}).

PPBR4^{SR}·hsCRE₂₄ complex whose K_d was 1.5 nM under identical conditions. Formation of the PPBR4^{SR}·hsCRE₂₄ complex was unaffected by high concentrations of poly (dIdC)·(dIdC) or a scrambled CRE site (NON), indicating that the high stability of PPBR4^{SR}·hsCRE₂₄ was not due primarily to nonspecific ionic interactions. Circular dichroism experiments indicated that like bZIP peptides,^{6,11} PPBR4^{SR} attained a fully α -helical conformation only in the presence of specific DNA.¹² Although others have described monopartite DNA recognition by basic segment peptides, the affinities reported have been only moderate (60 nM–3 μ M), and the complexes are stable only in very low ionic strength buffers.^{13,14} PPBR4^{SR} represents the first example of high affinity, monopartite, major groove recognition at physiological ionic strength.

To examine the contribution of hydrophobic core formation to PPBR4^{SR}·hsCRE₂₄ complex stability, we studied peptides G₂₇

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(2) The CD spectrum of PPBR4^{SR} was unchanged between 1 and 20 μ M, indicating that no detectable changes in secondary structure occurred in this range. Addition of hsCRE DNA significantly increased the α -helix content of PPBR4^{SR}; smaller changes were observed upon addition of hsCEBP DNA (see Supporting Information).

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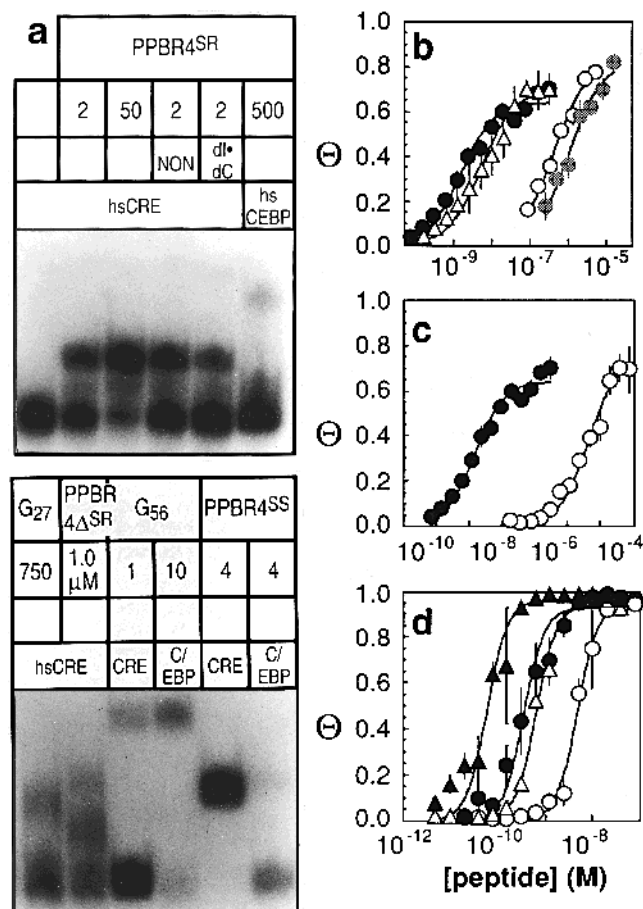


Figure 3. DNA recognition by miniature PPBR proteins. (A) Autoradiogram illustrating binding at the protein concentrations shown in nM (unless indicated otherwise) to ^{32}P DNA (AGTGGAGXCTCGTGC): hsCRE₂₄, X = ATGACAGCTA; hsCEBP₂₄, X = ATTGCAGCTA; CRE₂₄, X = ATGACGTCAT; CEBP₂₄, X = ATTGCGCAAT in the presence or absence of unlabeled, nonspecific DNA (NON, X = TAAGGCCTAT) or poly (dIdC)·(dIdC).^{30,31} (B) The fraction of hsCRE₂₄ bound (Θ) as a function of (●) PPBR₄^{SR}; (Δ) PPBR₂^{SR}; (○) G₂₇; or (gray circle) PPBR₄Δ^{SR} concentration. (C) The relative affinity of PPBR₄^{SR} for (●) hsCRE₂₄ and (○) hsCEBP₂₄. (D) The relative affinity of G₅₆ for (●) hsCRE₂₄, (○) hsCEBP₂₄, (▲) CRE₂₄ and (Δ) CEBP₂₄. Binding reactions were performed at 4 °C in phosphate-buffered saline [2.7 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄ (pH 7.3)] containing 5% glycerol, 1 mM EDTA, 0.4 mg/mL BSA, and 0.05% NP-40.

and PPBR₄Δ^{SR} (Figure 2). G₂₇ lacked the polyproline helix and turn, whereas PPBR₄Δ^{SR} contained D-tryptophan at position 4 and leucine at position 31. Modeling studies suggested that these substitutions would disrupt core formation by kinking the polyproline or the α-helix. The stabilities of the G₂₇·hsCRE₂₄ and PPBR₄Δ^{SR}·hsCRE₂₄ complexes were 3.1 and 3.2 kcal·mol⁻¹ lower, respectively, than that of PPBR₄^{SR}·hsCRE₂₄ complex (Figure 3). These data indicate that hydrophobic core formation stabilized the PPBR₄^{SR}·hsCRE₂₄ complex by as much as 3 kcal·mol⁻¹.

The sequence specificity of PPBR₄^{SR} was examined by comparing its affinity for hsCRE₂₄ to that for hsCEBP₂₄, a sequence containing the half-site recognized by C/EBP bZIP proteins (Figure 2).¹⁵ This half-site (ATTGC) differs from the CRE half-site (ATGAC) by two base pairs and provides an excellent measure of base pair specificity.^{16,17} PPBR₄^{SR} displayed

remarkable specificity for hsCRE₂₄ (Figure 3). The specificity ratio K_{rel} ($K_{\text{rel}} = K_{\text{d}}(\text{hsCRE})/K_{\text{d}}(\text{hsCEBP})$) describing preferred recognition of hsCRE₂₄ by PPBR₄^{SR} was 2600 ($\Delta\Delta G = -4.4$ kcal·mol⁻¹). By contrast, G₅₆, which comprised the bZIP element of GCN4, displayed low specificity. Specificity ratios of 118 and 180 were observed for binding of CRE₂₄ by G₅₆ in preference to CEBP₂₄ and hsCRE₂₄ in preference to hsCEBP₂₄, ($\Delta\Delta G = -2.6$ and -2.9 kcal·mol⁻¹), respectively. The relative specificities of G₅₆ and PPBR₄^{SR} were most recognizable when one considered the concentration of each protein required to bind one-half of the two DNAs: For PPBR₄^{SR}, this difference corresponded to a ratio of 2600, whereas for G₅₆, it corresponded to a ratio of 11. PPBR₄^{SR} more readily distinguished the two base pair difference between hsCRE₂₄ and hsCEBP₂₄ than G₅₆ distinguished CRE₂₄ from hsCEBP₂₄, two sequences that differed by six of ten base pairs. These comparisons emphasize that PPBR₄^{SR} was considerably more selective than was GCN4, the protein on which its design was based.

Recognition of a unique site in the human genome requires discrimination of a specific sequence of approximately 16 consecutive base pairs from all other possible sequences.¹⁸ While ligands with extremely high DNA affinities have been created from linear arrays of proteins^{19–22} or amino acids^{23–25} that individually possess defined sequence preferences, the ability to differentiate between closely related sequences remains a considerable challenge. Molecules whose design is based on multimerization typically tolerate, with minimal losses in affinity, target sites in which as many as half of the base pairs are changed from those in the intended target site.^{19,20,22,26} In addition, the long kinetic lifetimes of these complexes result in trapping of the molecules on nonintended targets.^{22,27} Indeed, the disulfide-linked dimer of PPBR₄, PPBR₄^{SS}, confirmed these limitations: PPBR₄^{SS} bound CRE₂₄ tightly ($K_{\text{d}} = 165$ pM) but differentiated CRE₂₄ from CEBP₂₄ with a moderate specificity ratio of 39. Monomeric PPBR₄^{SR}, by virtue of rapid equilibration with its preferred target site and high specificity, overcame the limitations of multimeric recognition.

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Supporting Information Available: Figure showing CD analysis and experimental details (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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