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SPECIFIC DNA BINDING PEPTIDE-DERIVATIZED SOLID SUPPORT

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Abstract. The basic region (amino acids 226-252) of the basic region/leucine zipper protein GCN4 has been synthesized and covalently attached to a column matrix. This peptide dimer-derivatized column binds to the AP-1 (5'-TGACTCA-3') and ATF/CREB (5'-TGACGTCA-3') DNA sites, which are the target sites of native GCN4.

Several protein motifs, including the helix-turn-helix (HTH), zinc finger, and basic region/leucine zipper (bZIP), have been found to be responsible for the high affinity and sequence specificity intrinsic to a number of DNA-binding proteins.[1] These structural motifs employ the α-helix for sequence-specific recognition of the DNA major groove. Examination of how α-helices interact with short DNA duplexes promises to be a starting point for understanding DNA recognition by full proteins.

In order to dissect specific recognition interactions between proteins and DNA, I constructed a column derivatized with the basic region dimer of yeast transcriptional activator GCN4, a homodimeric protein that employs the α-helical bZIP structure for specific DNA recognition. The leucine zipper mediates dimerization, and the basic region DNA binding.[2,3] Short peptide dimers comprising just the basic region of GCN4 were previously shown to retain the DNA-binding sequence specificity of native GCN4.[4,5] The basic region of GCN4 was synthesized and dimerized through a disulfide bond, rather than dimerization through the leucine zipper. These short peptide dimers were attached to a column matrix through diazotization to a tyrosine side chain, and the dimer-derivatized column binds to specific DNA sites (Figure 1).

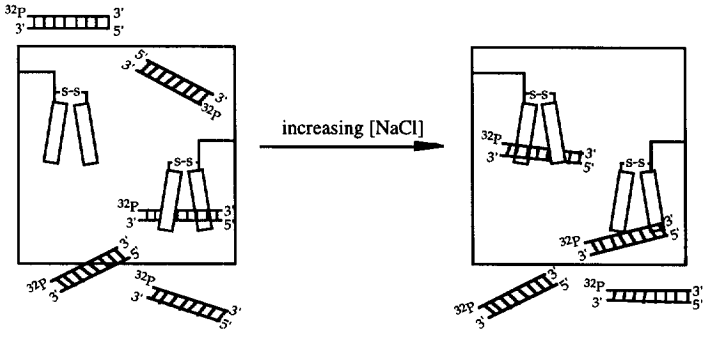

Figure 1. Schematic representation of the DNA selection process. At left is shown the column derivatized with the disulfide-linked basic region dimers. The radioactively labeled DNA library is applied to the column. Unbound DNAs are eluted. At right is shown elution by gradually increasing concentrations of salt.

Struhl and coworkers synthesized a column derivatized with native, expressed GCN4 homodimer, and demonstrated that it binds to specific DNA sequences.[6] In contrast, the work presented here allows for two major differences: (1) exploration of the minimal peptide motif required for specific DNA binding and (2) equally facile synthesis of heterodimeric and homodimeric peptides. This strategy for immobilization of peptides or molecules to a solid support can be generalized for use in other systems as well, including peptidomimetics and expressed proteins, and these derivatized columns have utility in strategies involving selection from random pools of ligands.

Peptide Synthesis

Previous work by Kim and coworkers demonstrated that disulfide-linked peptide dimers comprising the basic region of GCN4 and Gly-Gly-Cys (GGC) linker display DNA-binding capabilities very similar to native GCN4 (281 amino acids).[4,5] Two peptides suitable for immobilization to a solid support were therefore synthesized and purified (Figure 2).[7-9] One peptide, bGCN4(226-252)Cys, contains a GGC linker at the carboxyl terminus; glycines provide flexibility and the cysteine the means for dimerization.[4] The other peptide, bGCN4(226-252)Tyr, contains the additional Gly-Gly-Tyr (GGY) linker used in diazotization coupling with the amine-derivatized column matrix.[10,11] This peptide is first covalently attached to the column. bGCN4(226-252)Cys, which contains only the GGC linker, is then dimerized to the peptide monomer-derivatized column.

This strategy allows construction of heterodimeric peptides and enlarges the repertoire of possible protein-DNA interactions, as homodimers are generally restricted to binding (pseudo)palindromic DNA sites. Pellegrini and Ebright also synthesized short heterodimeric bZIP peptides, but dimerized through an N^{α}, N^{ε}-lysine linkage, where N^{α} and N^{ε} are differently protected.[12] Heterodimers were synthesized by deprotection of N^{α} followed by continued synthesis of one basic region peptide, then deprotection of N^{ε} and synthesis of the second peptide. These peptides can remain linked to resin after synthesis, and the resin-linked dimers can bind DNA. In contrast to Pellegrini and Ebright's work, the presented strategy provides two significant variations: (1) resin-linked heterodimers can be easily changed by reduction of the disulfide bond, followed by dimerization to a new peptide and (2) the use of resin-linked HPLC-purified peptides—for resin-linked peptides, Pellegrini and Ebright's strategy requires that peptides not be cleaved from resin and therefore, cannot be purified after synthesis.

GCN4 Sequences

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bZIP GCN4(226-281) DPAALKRARNTEAARRSRARKLQRMKQLEDKVEELLSKNYHLENEVARLKKLVGER

bGCN4(226-252)Tyr  DPAALKRARNTEAARRSRARKLQRMKQ-GGC-GGY
bGCN4(226-252)Cys  DPAALKRARNTEAARRSRARKLQRMKQ-GGC
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Figure 2. Sequences of the basic region of GCN4, amino acids 226-252. At top is shown the full bZIP sequence GCN4(226-281) for reference. The Cys and Tyr residues necessary for disulfide coupling and column attachment, respectively, are highlighted.

Increase in Peptide Helicity upon Specific DNA-Binding Monitored by Circular Dichroism

In the absence of bound DNA, bZIP basic regions are disordered, but helicity increases upon sequence-specific DNA binding.[4,13-16] To ascertain that these disulfide-linked synthesized peptides were capable of binding to specific DNA sites, circular dichroism (CD) studies were performed on the bGCN4(226-252)Cys

dimer. 5 micromolar peptide dimer showed some helicity (Figure 3), which was further increased upon addition of 5 μM duplex DNA containing the AP-1 sequence (5'-TGACTCA).

Peptide helicity may be induced by nonspecific DNA interactions. Johnson et al. demonstrated that nonspecific duplex $(dA)_{20}(dT)_{20}$ can serve as a template for induction of helicity in short peptides containing alanine and lysine.[17] However, CD measurements of bGCN4(226-252)Cys dimer incubated with nonspecific DNA showed little induced helicity, with helicity increases ranging from 4% to 8% between 4 °C and 22 °C (as measured at 222 nm). Equilibration of bGCN4(226-252)Cys with AP-1 duplex gains 18% to 37% helicity in the same temperature range. As a note, under these conditions of low salt (20 mM NaCl), more α-helical content was observed in bGCN4(226-252)Cys dimer standard (no DNA) than in similar experiments by Talanian et al.,[4] who used 100 mM NaCl; O'Neil et al.,[15,16] who used 150 mM NaCl; and Weiss et al.,[14] who used 200 mM KCl.

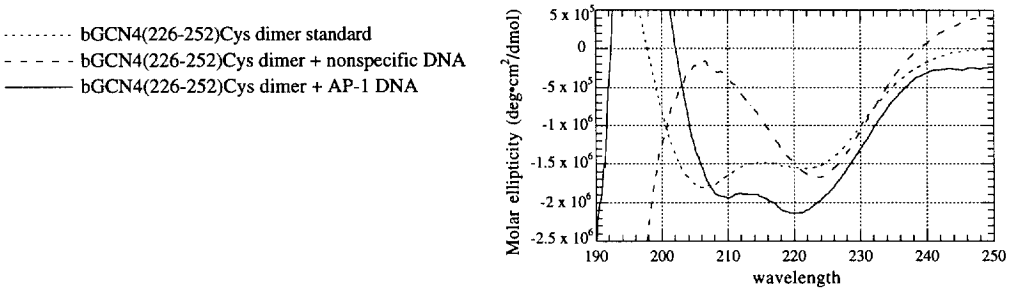

Figure 3. Helicity of bGCN4(226-252)Cys dimer standard versus helicity of peptide dimer incubated with nonspecific DNA or with AP-1 DNA site.[18] The CD spectrum of the nonspecific DNA control or the 53-mer AP-1 DNA control was substracted from the spectrum of the peptide dimer/nonspecific DNA complex or peptide dimer/AP-1 complex, respectively. 5 μM peptide dimer, 5 μM nonspecific DNA or AP-1, 20 mM phosphate buffer, pH 7.0, 20 mM NaCl, equilibration for 1h, 16 °C.

Derivatization of Solid Support with bGCN4(226-252)Tyr

The overall strategy for derivatization of solid support with peptide involves first linking one peptide monomer to the support by diazotization to a tyrosine side chain (Figure 4).[10,11] Disulfide linkage through cysteine side chains to the second peptide generates the peptide dimer-derivatized support.

The tyrosine-containing peptide bGCN4(226-252)Tyr was covalently attached to Affi-Gel 102 (Bio-Rad), which contains a primary amine linked via a hydrophilic linker at a loading of 12 μmol free amine/mL drained gel. As the aliphatic diazonium ion is highly unstable, the first reaction is to attach *m*-aminobenzoic acid to Affi-Gel by use of the water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC): the aromatic diazonium ion is stable, due to resonance, and can be used without isolation. The diazonium group was generated with sodium nitrite in acid, followed by in situ neutralization and addition of bGCN4(226-252)Tyr at pH 10 to generate a bright orange gel.

Quantitative assay[19] shows the *m*-diazobenzoyl group to be more reactive to coupling to tyrosine side chains than the *p*-diazobenzoyl group; *m*-diazobenzoyl was also found to be a more efficient coupling agent of daunomycin to bovine serum albumin than *p*- or *o*-diazobenzoyl.[20] Kinetics experiments[21] established that under these conditions, other amino acid side chains do not react with the diazonium ion: histidine does, however, react with the diazonium group at lower pH. Overall yield of peptide monomer-derivatized gel is ≥90%.

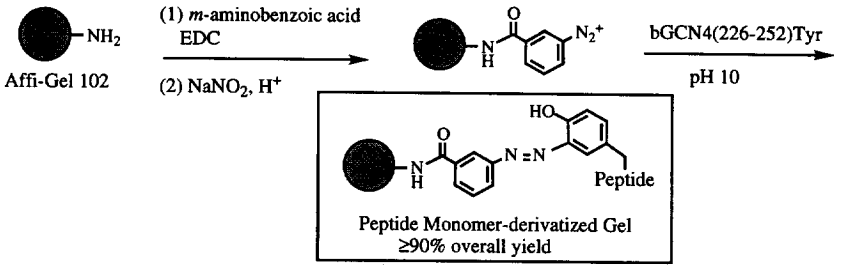

Figure 4. Covalent attachment of peptide monomer bGCN4(226-252)Tyr to solid support. Coupling of *m*-aminobenzoic acid to Affi-Gel followed by reaction of aromatic diazonium ion with tyrosine side chain on peptide.

Dimerization of Cysteines

The cysteine-containing peptides did not dimerize well under published conditions for similar peptides: glutathione redox buffer[22] or air oxidation in Tris buffer.[23] Instead, 2,2'-dithiobis(5-nitropyridine) (DTNP) was used to activate the thiol on the monomer-derivatized gel (Figure 5).[24,25] The activated thiol was then reacted with bGCN4(226-252)Cys to form the peptide dimer-derivatized gel, which was placed into a small fritted funnel to serve as the dimer-derivatized column. Reaction yields were assessed by measurement of 2-thio(5-nitropyridine) (TNP) concentration: ε_{386} = 14,000 $M^{-1} \cdot cm^{-1}$ for TNP.[26,27] Monitoring of TNP demonstrated that DTNP activation of cysteine thiol occurred stoichiometrically, whereas dimerization of bGCN4(226-252)Cys peptide with activated thiol occurred at ≥95%. The overall yield of peptide dimer-derivatized Affi-Gel is therefore ≥85%.

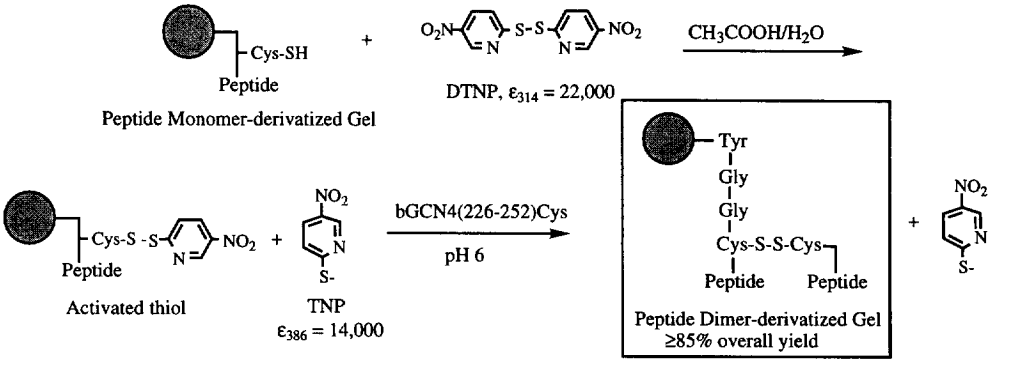

Figure 5. Activation of cysteine thiol by DTNP. Peptide monomer-derivatized gel is stirred with DTNP in acetic acid/water. Presence of TNP is determined by UV/vis.[27] The activated-thiol gel is then reacted with free peptide bGCN4(226-252)Cys in ammonium acetate buffer. Overall yield can be monitored by measuring TNP concentration.

The AP-1 DNA Sequence Binds Specifically to bGCN4 Dimer-derivatized Column

Duplex DNA containing the AP-1 site was radiolabeled with [α^{32}P]-dATP equilibrated on a column containing approximately 100 μL bGCN4 dimer-derivatized gel.[18] The column was eluted with aliquots of buffer containing increasing amounts of sodium chloride,[6] which disrupts noncovalent interactions between proteins and DNA. Unbound sequences are eluted at low salt concentrations, whereas retained sequences elute at higher salt.

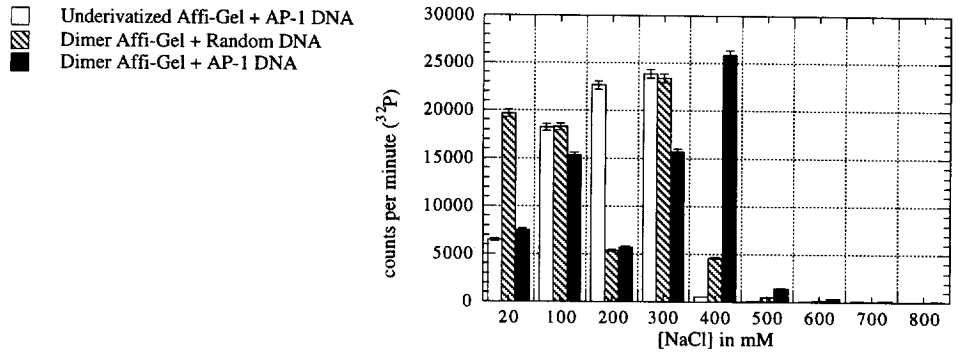

Figure 6. Incubation of radiolabeled AP-1 or random DNA with bGCN4 dimer-derivatized column. By 300 mM NaCl, nonspecifically bound DNA is eluted for both underivatized Affi-Gel equilibrated with AP-1 and dimer-derivatized Affi-Gel with random DNA. The dimer-derivatized column shows a strong peak of bound AP-1 DNA at 400 mM NaCl. Radioactive content of each aliquot was measured by scrintillation counting. Total amounts of radioactivity loaded onto each column were identical.

Plain, underivatized Affi-Gel retains AP-1 DNA by nonspecific interactions until elution at 300 mM NaCl (Figure 6, clear bars). The dimer-derivatized gel columns retain DNA until 300 mM NaCl as well, likely partly due to nonspecific interactions with the gel. Dimer Affi-Gel equilibrated with random DNA serves to examine nonspecific electrostatic effects between a positively charged basic peptide and negatively charged DNA; at 400 mM NaCl, a small amount of random DNA is retained by the column (Figure 6, hatched bars). Dimer Affi-Gel incubated with AP-1 DNA, however, shows strong binding at 400 mM NaCl. Struhl and coworkers achieved a very similar DNA elution profile with a native GCN4-Sepharose column.[6]

Conclusion

The bGCN4 dimer-derivatized column can be synthesized with reproducibly high yields and is capable of binding to the AP-1 and ATF/CREB DNA sites. Tyrosine-containing molecules—as well as histidine-containing molecules—can be readily attached to a solid support. Therefore, molecules other than peptides can also be derivatized with *p*-cresol or imidazole groups for facile diazotization reaction with a support carrying an aromatic amine. The derivatized solid support can be placed into a column or porous container ("tea bag") to enable facile selection from large libraries of soluble ligands. The work presented in this paper involved aqueous chemistry; originally however, carbodiimide coupling of *m*-aminobenzoic acid to Sepharose gel (Pharmacia) was performed in dichloromethane, and Sepharose did not suffer from exposure to organic solvent. This chemistry for immobilization of molecules via aqueous or organic means to solid support should prove particularly useful for strategies involving selection from large pools of ligands.

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18. The 53-mer AP-1 oligonucleotide template comprises 5'-atgcgcatgtggatccc-TCCGGATGACTCATTTTTTG-cgagctcatgcatatgc-3'; the 17-bp polymerase chain reaction (PCR) primers are in lower case, and the AP-1 site is underlined. Random/nonspecific DNA is comprised of the same PCR primers as for the AP-1 template, but flanking a randomized 20-bp region (N_{20}). Oligonucleotides were synthesized at the DNA Synthesis Facility, University of Pittsburgh. Duplex DNA was synthesized and radiolabeled by incubation of oligonucleotide template, primers, DNA polymerase, dNTP stock solutions, and [α^{32}P]-dATP. To dry DNA was added incubation buffer: 0.1 mg/mL bovine serum albumin (BSA, nuclease/protease-free, Sigma), 0.1 mg/mL tRNA (type XX, Sigma), 50 mM Tris, pH 8.0, 10 μg/mL gelatin, 1 mM EDTA, and 20 mM NaCl. Mixture was added to dimer-derivatized column, ≥1h, 4 °C, and eluted with buffer containing increasing concentrations of NaCl. Radioactivity was assessed by scintillation counting.
19. The basic protocol for the hydroxynaphthaldehyde assay was provided by Robert Kennedy, Pharmacia (Ragnarsson, U; et al. *Acta Chem. Scand.* **1970**, *24*, 2821). Hydroxynaphthaldehyde reacts with free primary amines. Model compounds were reacted with *m*-diazobenzoyl-derivatized gel: butylamine (lysine), ethanethiol (cysteine), 3-methyl indole (tryptophan), 4-methyl imidazole (histidine), *p*-cresol (tyrosine). Unreacted gel was then quenched with excess *N*-ethyl-ethylenediamine, which leaves a primary amine for reaction with hydroxynaphthaldehyde. Reaction was filtered and absorbance of supernatant was read: ε_{420} = 10,000 $M^{-1}\cdot cm^{-1}$.
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