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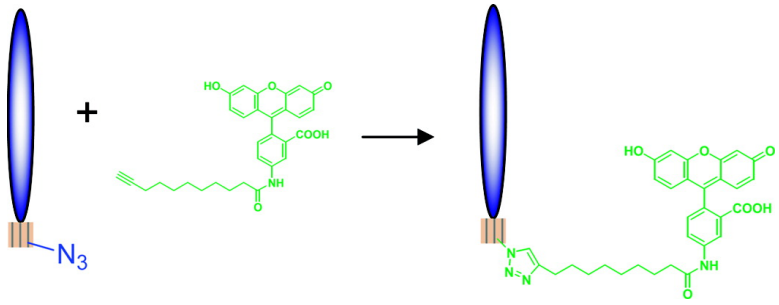
J. Am. Chem. Soc., **2004**, 126 (49), 15962-15963• DOI: 10.1021/ja045673m • Publication Date (Web): 17 November 2004



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Published on Web 11/17/2004

A Phage Display System with Unnatural Amino Acids

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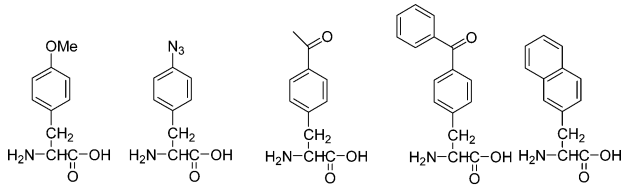
Phage display has proven very useful for the isolation of high-affinity ligands and receptors from large polypeptide libraries.[1] It has the advantages that large libraries can be easily generated by recombinant methods, library members can be amplified for iterative rounds of enrichment, and primary structure can be determined by DNA sequencing. However, in contrast to synthetic peptide libraries, phage displayed libraries are restricted to the common 20 amino acid building blocks, limiting the functional groups and structural motifs that can be explored. To expand the scope of this method, Noren and co-workers incorporated selenocysteine into phage displayed peptides using a natural selenocysteine opal suppressing tRNA.[2] Roberts et al. attempted to generalize this approach to peptide libraries containing other unnatural amino acids using in vitro mRNA display[3] with chemically aminoacylated amber suppressor tRNAs.[4] However, the generation of a large number of such tRNAs is difficult, and they are used stoichiometrically. Recently, we developed a method that allows one to genetically encode unnatural amino acids in both prokaryotic and eukaryotic organisms in response to nonsense and frameshift codons.[5−8] Over 30 unnatural amino acids have been selectively introduced into proteins with good efficiency and high fidelity. Here we show that this methodology can be used in conjunction with phage display as a general approach to the generation of polypeptide libraries containing unnatural amino acid building blocks.

Two plasmids, pDULE/CM and M13KE, are used to display polypeptides containing unnatural amino acids as fusions to the pIII protein of M13 filamentous phage. Plasmid pDULE/CM, which has a p15A origin, constitutively expresses a *Methanococcus jannaschii* amber suppressor $tRNA^{Tyr}$ (MjtRNA) and a mutant *M. jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) in *Escherichia coli*. This mutant MjTyrRS aminoacylates the amber suppressor tRNA with the desired unnatural amino acid. Growth of *E. coli* Top10 F′ harboring pDULE/CM (designated strain TTS) in the presence of the corresponding unnatural amino acid results in the incorporation of the unnatural amino acid at the site specified by the amber codon TAG. The second plasmid, M13KE, is a phage vector used for pentavalent N-terminal pIII display; a derivative, pM13KE-SBP, displaying a pIII fusion streptavidin binding peptide (SBP), AGXTLLAHPQ, was used in this study. The N-terminal AG sequence facilitates cleavage of the signal peptide. The third residue, X, encoded by amber nonsense codon TAG, designates the unnatural amino acid to be incorporated. Expression of the pIII fusion protein in *E. coli* strain TTS in the presence of the unnatural amino acid should afford viable phage that display the peptide containing the unnatural amino acid as a pIII fusion. To prepare the initial phage stocks, plasmid pM13KE-SBP was transformed into the *E. coli* XL1-Blue, a natural amber suppression strain that incorporates glutamine at residue X.

To examine the dependence of phage plaque formation on the presence of the unnatural amino acid, M13KE-SBP phage and M13KE wild-type phages were plated on *E. coli* TTS/RS **3** (RS **3** designates an aminoacyl tRNA synthetase specific for *p*-acetylphenylalanine **3)** cell lawns in the presence and absence of 2 mM *p*-acetylphenylalanine **3**. In the presence of the unnatural amino acid, both M13KE-SBP phage and M13KE wild-type phages formed normal-sized plaques after overnight incubation at 37 °C. However, in the absence of the unnatural amino acid, only M13KE wild-type phage formed plaques. No plaque formation was observable for M13KE-SBP phage. The M13KE-SBP phage yield in the natural glutaminyl amber suppressor strain XL1-Blue was 2×10^{11} plaque-forming units per milliliter of culture (PFU/mL). The yield of M13KE-SBP phage in *E. coli* TTS/RS **3** in the presence of 2 mM *p*-acetylphenylalanine **3** is comparable to that produced in XL1-Blue and is dependent on the presence of *p*-acetylphenylalanine **3**. In the presence of this unnatural amino acid the phage yield is 1.8×10^{11} PFU/mL; in the absence of the unnatural amino acid the phage yield is reduced by 81-fold. In a large-scale phage preparation, a difference of over 1000-fold was obtained. The phage yield experiments were carried out with five *E. coli* TTS/RS cell lines that incorporate five distinct unnatural amino acids: *O*-methyltyrosine, **1**,[5] *p*-azidophenylalanine **2**, *p*-acetylphenylalanine **3**,[10] *p*-benzoylphenylalanine **4**,[11] and 3-(2-naphthyl)alanine **5**.[12] Similar results (Table 1) were obtained in each case indicating that this display scheme is likely to be general for a large number of unnatural amino acids.

Table 1. Amino Acid (AA) Dependence of Phage Yields (PFU/mL)



	1	**2**	**3**	**4**	**5**
no AA	1.6×10^9	8×10^8	2.2×10^9	1.0×10^9	1.4×10^9
AA	1.2×10^{11}	1.0×10^{11}	1.8×10^{11}	1.6×10^{11}	2.0×10^{11}
ratio	75	125	81	160	143

Additional biochemical experiments were then carried out to further characterize this phage display system. M13KE-SBP phages were produced in *E. coli* TTS/RS **2** in the presence of 2 mM *p*-azidophenylalanine **2**. The resulting phages were then conjugated with the alkyne-derivatized fluorescein dye[13] **6** by a highly specific azide−alkyne [3 + 2] cycloaddition.[14] Phages prepared in XL1-Blue were used as a negative control. Approximately 10^{11} phages were used in conjugation reactions. The reactions were carried out at 4 °C for 16 h. Phage precipitated completely under the reported conditions [2 mM tris(carboxyethyl)phosphine (TCEP), 2 mM tris-(triazolyl) amine ligand, 2 mM fluorescein dye **6** and 1 mM $CuSO_4$ in potassium phosphate buffer (PB) at pH 8.0 with 5% *tert*-butyl alcohol as cosolvent].[15] Replacement of TCEP with Cu wire

10.1021/ja045673m CCC: $27.50

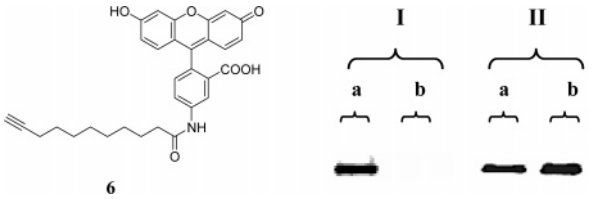


Figure 1. Alkyne derivatized fluorescein dye **6** and anti-fluorescein (I) and anti-pIII (II) Western blot analysis of [3 + 2] cycloaddition of M13KE-SBP phages: (a) phage prepared in TTS/RS **2**; (b) phage prepared in XL1-Blue.

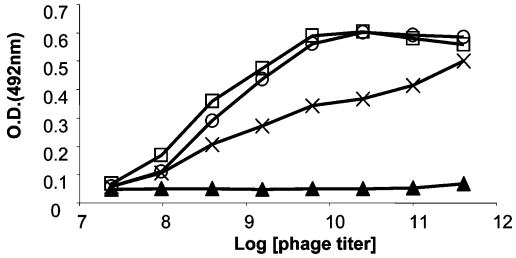


Figure 2. Phage streptavidin binding ELISA: (▲) M13KE; (□) M13KE-SBP phage prepared in TTS/RS**3**; (○) M13KE-SBP phage prepared in TTS/RS **2**; (×) M13KE-SBP phage prepared in XL1-Blue.

provided no improvement. Precipitation was minimized when diluted reagents were used (0.1 mM TCEP, 0.2 mM ligand, 0.2 mM fluorescein dye **6** and 0.1 mM $CuSO_4$ in PB buffer at pH 8.0).[16] After conjugation, the reaction mixture was dialyzed and subjected to SDS-PAGE and Western blot analysis (Figure 1). The fluorescein conjugate was detected as a single band only in the case of phage produced in TTS/RS **2** supplemented with 2 mM *p*-azidophenylalanine **2**. This band was further identified as the pIII minor coat protein by anti-pIII Western blot analysis. These results demonstrate that the unnatural amino acid is incorporated specifically into the pIII coat protein of the unnatural phage.

To demonstrate that the mutant streptavidin binding peptide presented on the N-terminus of the pIII protein is functional, we carried out an enzyme-linked immunosorbent assay (ELISA) using M13KE-SBP phage prepared in TTS/RS **2** and TTS/RS **3** cells supplemented with 2 mM *p*-azidophenylalanine **2** and *p*-acetylphenylalanine **3**, respectively (Figure 2). M13KE-SBP phage prepared in XL1-Blue served as a positive control, while wild-type M13KE phage served as a negative control. Streptavidin coated titer plates were blocked with 4% BSA and incubated with phage. Figure 2 shows that M13KE-SBP phage prepared in TTS/*p*-azidophenylalanine **2** and TTS/*p*-acetylphenylalanine **3** bind to streptavidin more strongly than the positive control phage prepared in XL1-Blue. This increase in observed affinity might result from increased binding affinity or proteolytic stability of the displayed peptide containing the unnatural amino acids. In a model phage selection experiment, a similar number of M13KE-SBP phage prepared in TTS/RS **3** and M13KE wild-type phage were incubated in two separate streptavidin coated wells. After iterative washing, the bound phage were eluted from the solid support with biotin and titered. The recovery rate of M13KE-SBP phage prepared in TTS/RS **3** is 9×10^3 fold over that of M13KE wild-type phage. These experimental results show that the mutant SBP is displayed on phage and is functional.

The generalization of phage display to include unnatural amino acids should significantly increase the scope of phage display technology. For example, the incorporation unnatural amino acids into phage-displayed polypeptides may lead to increased binding affinity and specificity, conformationally constrained backbones and side chains, and enhanced proteolytic stability. Unnatural amino acids may also provide reactive sites for the conjugation of nonpeptidic molecules as well as photoaffinity labels for the identification of orphan ligands or receptors. Finally, this methodology should also be applicable to other display formats such as ribosome and yeast display.

Acknowledgment. This work was supported by the National Institutes of Health (Grant No. GM56528). We thank the Sharpless group for providing us with the tris(triazolyl)amine ligand. We thank Dr. Alexander Deiters for providing the alkyne derivatized fluorescein dye **6**.

Supporting Information Available: Experimental details for vector constructions, the preparation of phage with unnatural amino acids, the cycloaddition reaction, the phage ELISA, and the phage enrichment. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA045673M