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Protocol can be downloaded from:
http://www.biochemistry.ucla.edu/biochem/Faculty/Hubbell/

Protocol for site-specific attachment of proteins containing *p*-AcF or *p*-AzF unnatural amino acids

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Materials:

Table 1: Estimated cost of relevant reagents used for incorporation of unnatural amino acids and subsequent attachment to solid support

Product	Vendor	Amount	Price (US $)	Amount used
p-Acetyl phenylalanine	SynChem	25 grams	1695	0.25 g per liter of *E. coli* cultures
p-Azido phenylalanine	SynChem	25 grams	2795	0.25 g per liter of *E. coli* cultures
DBCO-sulfo-link biotin	Click chemistry tools	100 mg	1095	1 mg per 3 mg of protein
DBCO-NHS ester	Click chemistry tools	100 mg	245	8 mg per ml of resin
N-(aminoacetyl)-N'-(D-biotinolyl)hydrazine, TFA	Invitrogen	100 mg	1710	1 mg per 2 mg of protein
CNBr-Sepharose	GE Life Sciences	15 grams	377	
High-capacity streptavidin agarose	Pierce	10 ml	682	50 - 100 ul per mutant

Protocol:

1. Expression and purification of proteins containing unnatural amino acids. For generation of protein containing unnatural amino acids, introduce the amber (TAG) stop codon at the site of interest using the QuikChange Site-Directed mutagenesis method (Stratagene). For unnatural amino acid incorporation in *E. coli*, two plasmids are required, one for expression of the gene of interest (containing the TAG codon) and a second that contains the orthogonal tRNA and aminoacyl-tRNA synthetase pair. For introduction of *p*-AcF the pSUPAR or pEVOL-*p*-AcF plasmid can be used. For introduction of *p*-AzF, the plasmid pEVOL-*p*-AzF should be used

instead. Unnatural amino acids *p*-acetylphenlyalanine and *p*-azidophenylalanine can be obtained from SynChem (Elk Grove Village, IL). Briefly, for protein expression, *E. coli* BL21(DE3) cells (Stratagene) are cotransformed with the vector containing your favorite gene and the vector containing the synthetase specific for the unnatural amino acid, and then plated onto LB-agar plates containing ampicillin and chloramphenicol for selection (100µg/mL ampicillin and 34 µg/mL chloramphenicol). For efficient cotransformation, use 200-300 ng of each vector. After overnight incubation at 37°C, select single colonies and inoculate into 20 mL of starter LB medium containing the aforementioned antibiotics and then grow overnight at 37 °C in a shaking incubator. The following day, inoculate the starter culture into 1-L of LB medium containing the aforementioned antibiotics and grow at 37°C in a shaking incubator to an OD_{600} of 0.75. Add 0.25 g of the unnatural amino acid and induce expression with 1mM isopropyl-β-D-thiogalactopyranoside and 0.02% L-arabinose, shake overnight at 30 °C, and then harvest by centrifugation. Purify the protein of interest according to your favorite protocol.

It should be noted that for purification of protein containing *p*-AzF, the azide group can be partially reduced to an amine during bacterial expression and by reducing agents such as DTT, BME, and TCEP that are often used to maintain cysteine residues in a reduced state. Thus, any purification procedure that required reducing agents such as DTT or BME should be adapted accordingly to minimize reduction of the azide during purification. The figure below shows the *in vitro* reduction of *p*-AzF by 5 mM TCEP (Figure adapted from *López et al.* 2014).

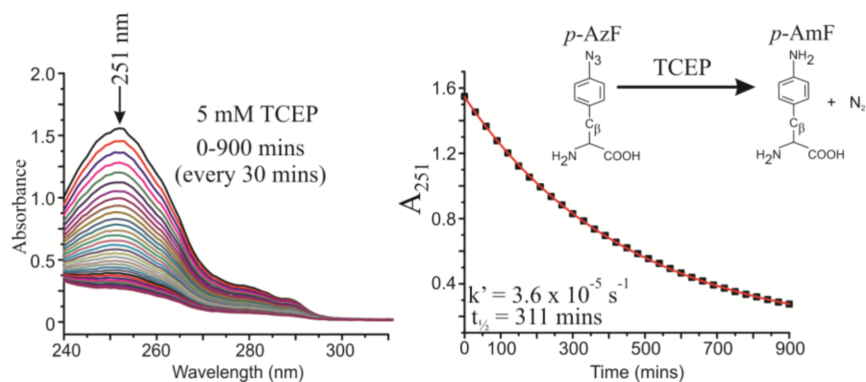
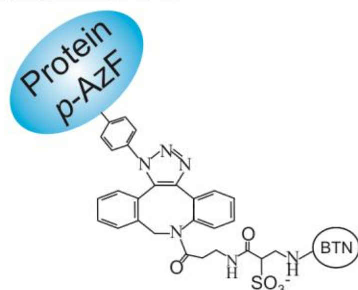


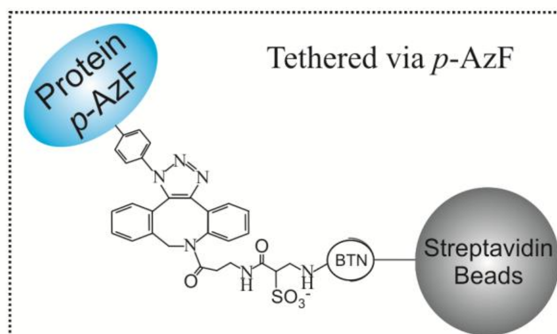
Figure 1. Conversion of *p*-AzF to *p*-AmF by 5 mM TCEP. See details of experimental conditions in *López et al.* 2014

Steps 2 (or 3) and 4 are for attachment via bioaffinity

Bioaffinity

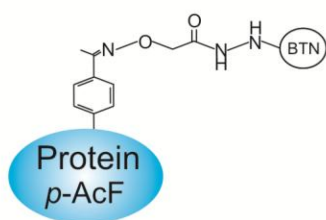
Scheme A





Streptavidin beads

Scheme B



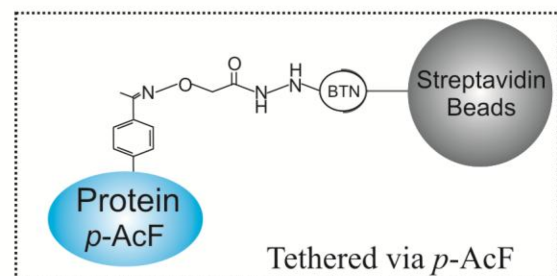


Figure 2. Strategy used for site-specific tethering using bioaffinity for proteins containing the indicated unnatural amino acids. Figure adapted from *López et al.* 2014

2. Biotinylation of proteins bearing *p*-AzF (Scheme A). Prepare a stock solution of 10 mM of DBCO-biotin prepared in DMF or DMSO and used immediately or stored at -20 °C. A 10-20-fold molar excess of DBCO-biotin should be added to 50-100 μM of protein bearing *p*-AzF in PBS (pH 7.2) and incubate the mixture at room temperature for 2-4 hours or at 4 °C overnight. Remove excess reagent by repeated washes with PBS (pH 7.2) buffer using a 15-ml Amicon Ultra concentrator (10 kDa MWCO). For spin labeling purposes, the spin labeling step should be done prior to incubation with DBCO-biotin to prevent thiol-yne addition.

3. Biotinylation of proteins bearing *p*-AcF (Scheme B). Mix purified mutants bearing *p*-AcF in a buffer consisting of 50 mM sodium phosphate, 25 mM NaCl at pH 4.0 with 10-fold molar excess of a freshly made solution of N-(aminooxyacetyl)-N'-(D-biotinoyl) hydrazine trifluoroacetic acid salt. Incubate the mixture overnight at 37 °C with nutation to yield the biotinylated protein. Remove excess reagent by three 15-ml washes with PBS buffer (100 mM phosphate, 150 mM NaCl) at pH 7.2, using a 15-ml Amicon Ultra concentrator (10 kDa MWCO).

4. Tethering biotinylated proteins to high-capacity streptavidin beads. Equilibrate the desired quantity of Strepavidin beads in PBS buffer (pH 7.2) and wash 3X by centrifugation with a 5-fold excess of the same buffer. After the final wash and removal of the supernatant, add the biotinylated protein to the beads in an amount equivalent to the stated capacity of the beads and mix the suspension at room temperature for at least 2 hours at room temperature or at 4 °C overnight. Remove the supernatant and wash the beads with final buffer. For EPR studies of T4L, the final buffer consists of 50 mM MOPS, 25 mM NaCl at pH 6.8. Determine the coupling efficiency using the A_{280nm} of the supernatant and washes and compare to that of the protein solution added to the beads. The attached protein is now ready for EPR measurements.

Steps 5 and 6 are for direct covalent coupling of proteins containing *p*-AzF to DBCO-Sepharose beads

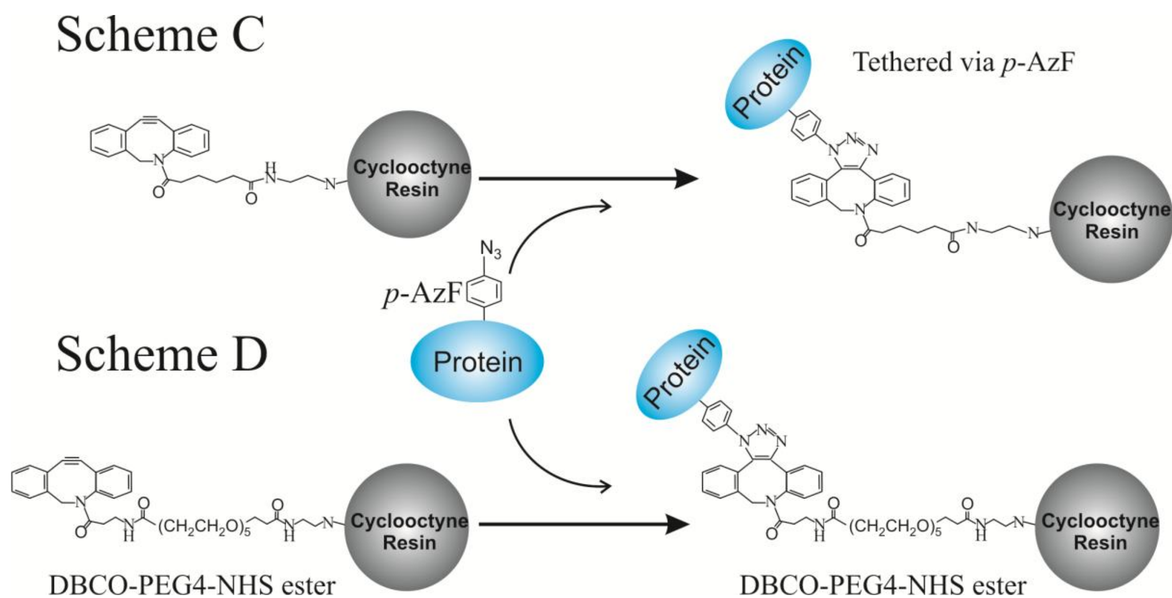


Figure 3. Strategy for direct covalent attachment for proteins containing *p*-AzF. Figure adapted from *López et al.* 2014

5. Functionalization of CNBr-Sepharose 4B beads with DBCO. Resuspend lyophilized CNBr-Sepharose 4B beads in 10 mM HCl at pH 2, mix for about 20 minutes, and then wash with a total volume of 100-200 ml of 10 mM HCl at pH 2 as suggested by the manufacturer. Equilibrate the beads were with coupling buffer consisting of 0.1 M NaHCO3, 0.5 M NaCl, at pH 8.3 and add ethylenediamine to a final concentration of 1 M to generate Amino-Sepharose resin. Incubate the suspension for at least 2 hours at room temperature with constant mixing. Remove excess ethylenediamine by repeated washes with at least 10 medium volumes of coupling buffer. Inactivate any remaining cyanate-ester group by incubating with blocking buffer (0.1 M Tris-Cl at pH 8.0) for 2 hours at room temperature. Wash the prepared amino-Sepharose with 3 cycles of high pH (0.1 M Tris-Cl, 0.5 M NaCl, pH 8.0) and low pH (0.1 M sodium

acetate, 0.5 M NaCl, pH 4.0) buffers to remove any traces of ethylenediamine that may be nonspecifically bound to the beads. For long-term storage, store the resin in 20% ethanol.

For functionalization with DBCO, pre-equilibrate the amino-Sepharose beads with PBS (pH 7.2), then add a freshly prepared 10 mM solution of DBCO-sulfo-NHS (**Scheme C**) or DBCO-PEG4-NHS (**Scheme D**) and incubate overnight at room temperature with nutation. Unreacted DBCO-NHS should be removed and inactivated via repeated washes with buffer consisting of 0.1 M Tris-Cl, 0.5 M NaCl at pH 8. Wash the resin 3 times with alternating high pH buffer (pH 8) – low pH buffer (pH 4) cycles to remove any nonspecifically bound reactant. Use the washed DBCO-Sepharose immediately (proceed to step 6) or store in 20% ethanol.

6. Tethering proteins bearing *p*-AzF on DBCO beads. Equilibrate the desired quantity of DBCO-Sepharose beads with PBS (pH 7.2), and add the protein bearing the *p*-AzF for direct coupling. Incubate the mixture overnight at room temperature or at 4 °C. After incubation, remove the supernatant and any uncoupled protein by washing with several medium volumes of final buffer. For T4L the final buffer consists of 50 mM MOPS, 25 mM NaCl at pH 6.8. Determine binding efficiency using A_{280nm} measurements of the supernatant and washes. The attached protein is now ready for EPR measurements.