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

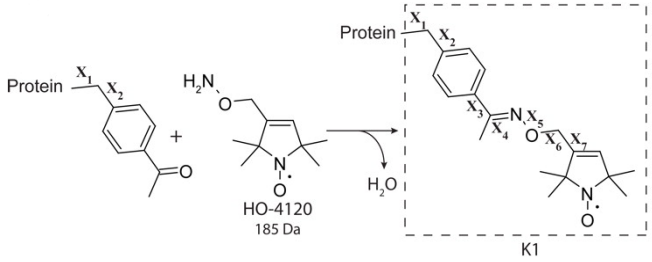
Expression of recombinant proteins containing unnatural amino acids in *E. coli* and spin labeling with K1 and T1

Note: For expression and purification of T4 lysozyme mutants containing *p*-AcF, follow the protocol published in *Fleissner et al.*[1] Expression of T4 lysozyme mutants containing *p*-AzF is described in *Kálái et al.*[2] The general protocol described below is for introduction of unnatural amino acids and subsequent spin labeling of any recombinant protein expressed in *E. coli*.

Generate amber (TAG) mutant 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 specific for the unnatural amino acid. For introduction of *p*-AcF the pSUPAR plasmid is used.[3] For introduction of *p*-AzF, the plasmid pEVOL-*p*-AzF should be used instead.[2] Unnatural amino acids *p*-acetylphenlyalanine and *p*-azidophenylalanine can be obtained SynChem (Elk Grove Village, IL). Briefly, for protein expression, BL21(DE3) *E. coli* (Stratagene) are cotransformed with the vector containing your favorite gene and pSUPAR or pEVOL-p-AzF, 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 20mL of 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 0.5g of the unnatural amino acid and grow at 37°C in a shaking incubator to an OD_{600} of 0.5. Induce the cultures 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.

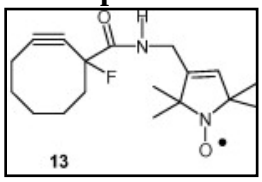
To spin label the purified protein containing either *p*-AcF or *p*-AzF follow the protocols described by Fleissner[4] and Kálái[2], respectively. Briefly, for spin labeling *via* the ketone group of *p*-AcF, pass the purify protein over a desalting column (HiPrep 26/10; GE Healthcare) equilibrated with labeling buffer. The labeling buffer should consist of 50mM monobasic sodium phosphate and 25mM NaCl at pH 4.0. After elution, concentrate the eluted protein to ~ 0.1–1 mM and then react with a 10-fold molar excess of HO-4120 for 12–48 h at 37 °C. For unstable proteins, spin labeling at pH ~ 7 can be done in the presence of the catalyst methoxyaniline as described by Fleissner et al.[1] Remove excess spin reagent by using the above-mentioned desalting column or by washing with an Amicon concetrator using the final EPR buffer. Concentrate the K1-labeled protein prior to EPR measurements.

K1-labeled protein (taken from Fleissner et al.[1])

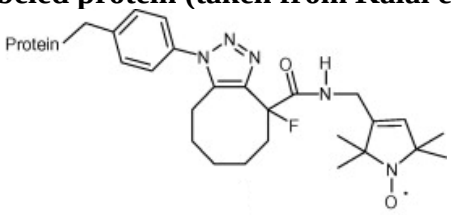


For spin labeling with an azido-specific label, a fivefold molar excess of compound **13** (shown below) should be added to the purified protein in buffer consisting of 50mM phosphate, 0.2M NaCl, 0.1mM EDTA at pH 7.6. Allow the resulting mixture to react overnight at ambient temperature or at 4 °C and remove unreacted spin label using a desalting column or an Amicon concentrator. Concentrate the T1-labeled protein prior to EPR measurements.

Compound 13



T1-labeled protein (taken from Kálái et al.[2])



References:

(1) Fleissner, M. R.; Brustad, E. M.; Kalai, T.; Altenbach, C.; Cascio, D.; Peters, F. B.; Hideg, K.; Peuker, S.; Schultz, P. G.; Hubbell, W. L. *Proceedings of the National Academy of Sciences of the United States of America* **2009**, *106*, 21637.

(2) Kálai, T.; Fleissner, M. R.; Jekő, J.; Hubbell, W. L.; Hideg, K. *Tetrahedron Letters* **2011**, *52*, 2747.

(3) Brustad, E. M.; Lemke, E. A.; Schultz, P. G.; Deniz, A. A. *Journal of the American Chemical Society* **2008**, *130*, 17664.

(4) Fleissner, M. R.; Cascio, D.; Hubbell, W. L. *Protein science : a publication of the Protein Society* **2009**, *18*, 893.