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Modification of Aniline Containing Proteins Using an Oxidative Coupling Strategy

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Protein bioconjugation strategies largely rely on the reaction of nucleophilic amino acid side chains with properly matched electrophiles.[1] As the selectivity of these reactions is primarily driven by the frequency of a particular residue on a protein surface, labeling approaches commonly target more rarely occurring amino acids, such as cysteines,[1] tryptophans,[2] and solvent accessible tyrosines,[3] when positional control is required. Despite these options, however, there remain many situations in which a unique residue is not available, limiting one's ability to achieve protein modification in a single location. Recently available techniques[4] for the incorporation of unnatural amino acids provide a powerful solution to this problem, as they allow unique chemical handles to be introduced and modified with reactions that disregard native functionality. Several of these strategies have been employed in the biological context, including the condensation of ketones with hydrazides and alkoxyamines,[5] the [3 + 2] cycloaddition of alkynes and azides to form triazoles,[6] and the formation of amides through a modified Staudinger reduction of azides with triarylphosphines.[7] Each of these methods is drawn from an important set of reactions that proceed in aqueous solution with excellent functional group tolerance.[8]

To add to this group, we have developed a new reactive pair based on the oxidative coupling of anilines. These functional groups are particularly attractive targets for selective bioconjugation in light of recent success for the incorporation of 4-aminophenylalanine into proteins.[9] Groundbreaking work by the Schultz lab has led to the evolution of a bacterium that can both biosynthesize this amino acid and carry it through protein translation. To target this side chain, we report herein a rapid, chemoselective, and high yielding reaction that couples new functionality to anilines under conditions mild enough to preserve the function of most biomolecules.

The development of this reaction began with the observation that *N*-acyl phenylenediamine derivative **1** trimerizes cleanly under oxidative conditions in aqueous solution, ultimately forming highly stable dye molecule **2** within minutes, Figure 1a.[10] To develop a two-component version of this reaction, this self-coupling pathway was first eliminated through the addition of two alkyl groups to the free phenylenediamine nitrogen, yielding **3**. Upon the addition of an oxidant, such as $NaIO_4$, little reaction occurs for **3** alone; however, this group still undergoes rapid coupling with primary anilines (such as **4**). The alkyl substituents of **3** also block the second aniline addition that would lead to three-component products. Instead, the final product results from nucleophilic addition of water to yield **5a** after subsequent oxidation (Figure 1b).[11] This "A + B" analog of **2** has similar stability, showing no degradation from pH 2 to pH 11 over 12 h. When carried out in $H_2{}^{18}O$, the corresponding product mass increased by 2 amu. Interestingly, the carbonyl group of **5b** showed no isotope exchange over a 24 h period when returned to $H_2{}^{16}O$, further confirming the high degree of stability toward hydrolysis. In addition to product **5a**, minimal amounts of aniline dimer (<10%) were also isolated.[11]

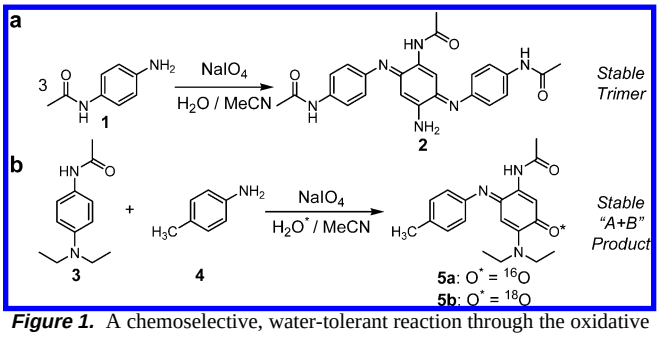

Figure 1. A chemoselective, water-tolerant reaction through the oxidative coupling of phenylenediamine derivatives **3** to anilines **4**.

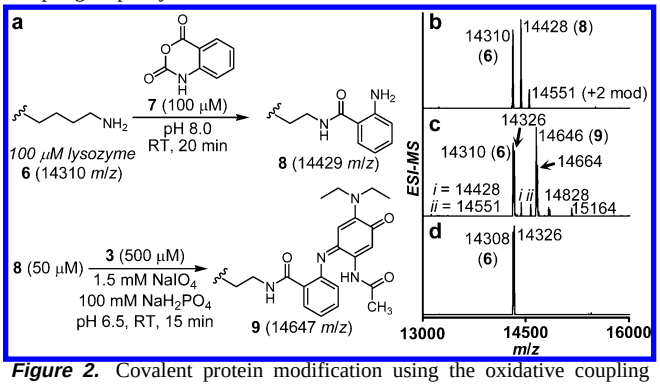

Figure 2. Covalent protein modification using the oxidative coupling reaction. (a) Anilines were introduced through the reaction of lysine residues with isatoic anhydride **7**. These groups were modified through exposure to **3** and $NaIO_4$. (b) Aniline-labeled lysozyme **8** was analyzed using ESI-MS. A small amount of doubly-acylated product can be seen at 14551 *m*/*z*. (c) Following treatment with **3** and $NaIO_4$, species **8** was converted to coupling product **9**. (d) No coupling products were observed upon exposure of unmodified lysozyme (**6**) to analogous reaction conditions. The new peak at 14326 *m*/*z* arises from methionine or cysteine oxidation by the periodate.

To evaluate the selectivity of this reaction for protein modification, an analog of **4** was coupled to lysine residues on lysozyme **6**. This was conveniently achieved through exposure to isatoic anhydride **7** at pH 8.0 for 20 min, Figure 2a. ESI-MS indicated 50% conversion to adduct **8**, with a small amount of doubly modified protein also present (Figure 2b).[11]

Oxidative coupling was achieved by exposing a 50 μM solution of **8** to 500 μM **3** and 1.5 mM $NaIO_4$ in pH 6.5 phosphate buffer for 15 min (Figure 2c). The anticipated mass adduct, corresponding to compound **9**, was observed with a high level of conversion.[12] For unlabeled lysozyme, no addition of **3** was observed under analogous conditions, indicating that none of the natural amino-acid side chains participated in the coupling reaction (Figure 2d). The reaction was accompanied by the oxidation of sulfur-containing side chains, which occurred to the same extent with or without the aniline or phenylenediamine components. In some reports of N-terminal serine oxidation with $NaIO_4$, methionine-containing buffers and/or carefully controlled quenches have been used to

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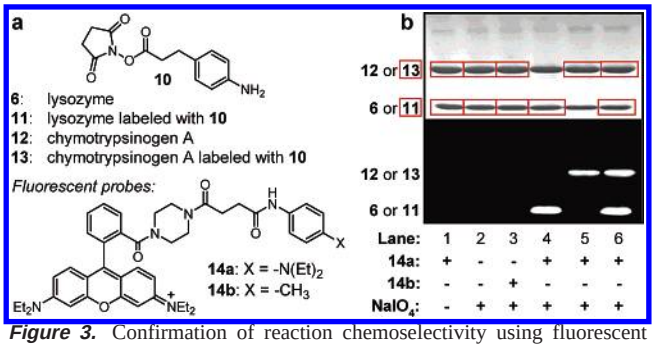

Figure 3. Confirmation of reaction chemoselectivity using fluorescent probes. (a) Samples were prepared by mixing lysozyme and chymotrypsinogen A. In some cases, these proteins had been labeled with anilines using reagent **10** (these samples are boxed in red in graphic b). The protein mixtures were then combined with $NaIO_4$ and fluorescent rhodamines **14a** or **14b**. (b) SDS-PAGE, followed by visualization with fluorescence imaging (bottom) and Coomassie staining (top), indicating the samples in which successful labeling had occurred.

minimize sulfur oxidation.[13] To date, these approaches have not been successful with our aniline coupling strategy. We are currently evaluating alternative oxidants to minimize these side reactions.[14] Similar to the small molecule studies, a small amount (3−8%) of protein crosslinking was detected in some instances.

The fluorescent labeling of uniquely functionalized proteins has proven useful for reactive profiling studies.[15] To explore the feasibility of the oxidative coupling reaction for this purpose, samples of lysozyme (**6**) and chymotrypsinogen A (**12**) were labeled with NHS-ester **10**, Figure 3a. Mixtures of the labeled and unlabeled proteins were then exposed to fluorescent dialkylphenylenediamine **14a** (250 μM) and $NaIO_4$ (1.5 mM). SDS-PAGE analysis indicated that only the aniline-bearing proteins participated in the reaction (Figure 3b, lanes 4−6). Additional control experiments confirmed that no reaction occurred in the absence of the oxidant (lane 1) or aniline component (lanes 2−3). To test the stability of the reaction product, a labeled sample of lysozyme was subjected to a series of conditions for 24 h and then analyzed. No losses in fluorescence were observed from pH 4−10, or in the presence of $NaIO_4$, reductants such as glutathione and dithionite, or nucleophilic reagents such as hydrazine or benzyloxyamine.[11]

As a convenient way to confirm the overall conversion of the reaction, a native chemical ligation strategy[16] was used to install a single aniline at the C-terminus of the green fluorescent protein (A206K eGFP) according to Figure 4.[16b,17] Upon exposure of a 50 μM solution of the aniline-labeled protein (**17**) to fluorescent probe **14a** (250 μM) and $NaIO_4$ (1 mM), 82% conversion was obtained in 2 h, as measured by UV−vis analysis.[11] When exposed to poly(ethylene glycol)-substituted phenylenediamine derivative **18**, 45% conversion to the singly labeled protein conjugate was observed (determined by optical densitometry after staining) (Figure 4b).

In summary, we have developed an efficient protein modification reaction that targets a bioorthogonal functional group under mild reaction conditions. This strategy features excellent chemoselecitvity, rapid coupling rates in buffered aqueous solution, and product stability over a wide range of conditions. Alternative activation strategies and coupling partners are under development, as are studies to determine the specific mechanisms by which these reactions proceed.

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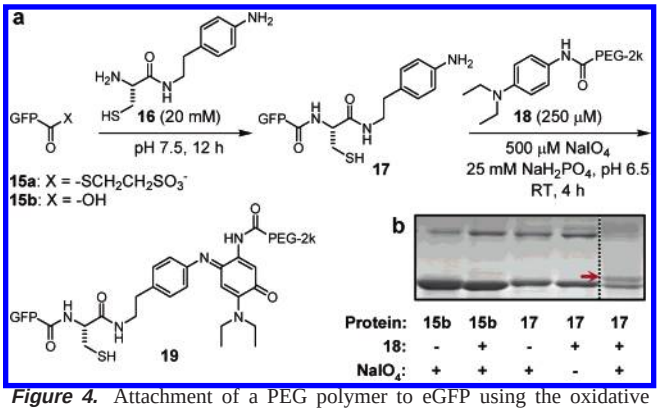

Figure 4. Attachment of a PEG polymer to eGFP using the oxidative coupling strategy. (a) A single aniline group was introduced through a native chemical ligation with **16**. (b) Following exposure to **18** and $NaIO_4$, a single PEG conjugate (indicated by the arrow) could be seen for **17**. The upper bands in the gel arise because of protein aggregation and are independent of the reaction conditions.

Supporting Information Available: Experimental procedures and characterization data for all intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996.
(2) Antos, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256−10257.
(3) (a) Fancy, D. A.; Denison, C.; Kim, K.; Xie, Y. Q.; Holdeman, T.; Amini, F.; Kodadek, T. *Chem. Biol.* **2000**, *7*, 697−708. (b) Hooker, J. M.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 3718−3719. (c) Schlick, T. L.; Ding, Z. B.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127*, 3718−3723. (d) Meunier, S.; Strable, E.; Finn, M. G. *Chem. Biol.* **2004**, *11*, 319−326. (e) Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 15942−15943. (f) Tilley, S. D.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128*, 1080−1081.
(4) For general reviews, see (a) Wang, L.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2005**, *44*, 34−66. (b) Link, A. J.; Mock, M. L.; Tirrell, D. A. *Curr. Opin. Biotech.* **2003**, *14*, 603−609.
(5) (a) Cornish, V. W.; Hahn, K. M.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 8150−8151. (b) Zhang, Z. W.; Smith, B. A. C.; Wang, L.; Brock, A.; Cho, C.; Schultz, P. G. *Biochemistry* **2003**, *42*, 6735−6746.
(6) (a) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192−3193. (b) Sen Gupta, S.; Kuzelka, J.; Singh, P.; Lewis, W. G.; Manchester, M.; Finn, M. G. *Bioconj. Chem.* **2005**, *16*, 1572−1579. (c) Deiters, A.; Cropp, T. A.; Mukherji, M.; Chin, J. W.; Anderson, J. C.; Schultz, P. G. *J. Am. Chem. Soc.* **2003**, *125*, 11782−11783. (d) Link, A. J.; Tirrell, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 11164−11165. (e) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046−15047.
(7) (a) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010. (b) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Nat. Acad. Sci. U.S.A.* **2002**, *99*, 19−24. (c) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430*, 873−877. (d) Nilsson, B. L.; Hondal, R. J.; Soellner, M. B.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 5268−5269.
(8) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004−2021.
(9) Mehl, R. A.; Anderson, J. C.; Santoro, S. W.; Wang, L.; Martin, A. B.; King, D. S.; Horn, D. M.; Schultz, P. G. *J. Am. Chem. Soc.* **2003**, *125*, 935−939.
(10) (a) Bandrowski, E. *Chem. Ber.* **1894**, *27*, 480. (b) Ritter, J. J.; Schmitz, G. H. *J. Am. Chem. Soc.* **1929**, *51*, 1587−1589. (c) Corbett, J. F. *J. Chem. Soc. B* **1969**, *818*. (d) Blake, A. J.; Hubberstey, P.; Quinlan, D. J. *Acta Crystallogr., Sect. C: Cryst. Struct. Comm.* **1996**, *52*, 1774−1776.
(11) See Supporting Information for mechanisms, spectra, and details.
(12) We have found that the phenylenediamine group can be attached to the protein and coupled to aniline reagents with the same chemoselectivity.
(13) For examples see (a) Chen, J. H.; Zeng, W. G.; Offord, R.; Rose, K. *Bioconjugate Chem.* **2003**, *14*, 614−618. (b) Zhang, L. S.; Tam, J. P. *Anal. Biochem.* **1996**, *233*, 87−93.
(14) We have already found that $(NH_4)_2Ce(NO_3)_6$ can be used with similar success, allowing improved compatibility with glycoprotein substrates.
(15) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686−4687.
(16) (a) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779. (b) Tolbert, T. J.; Wong, C. H. *J. Am. Chem. Soc.* **2000**, *122*, 5421−5428.
(17) (a) Tsien, Y. *Annu. Rev. Biochem.* **1998**, *67*, 509−544. (b) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906−918.

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