Simple method for selective bioconjugation of native proteins

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Whether it is for pharmaceuticals, or imaging, or proteomics, many scientists are interested in finding better ways to chemically tweak proteins. However, proteins are chemically complex, and targeting one particular place on the protein surface without also chemically modifying the surrounding amino acids can be tricky.

Ways to work around this problem include genetically engineering parts of the protein, but this can lower proteins expression levels, or devising a reaction scheme that is specific to a particular target on a protein; however, this is often complex, low yielding, and not scalable. James I. MacDonald, Henrik K. Munch, Troy Moore, and Matthew B. Francis of the University of California at Berkeley may have a solution. They have devised a general method for site-specific modification of the N-terminal portion of the protein that is mild, versatile, scalable, and does not require genetic engineering. Their work is presented in *Nature Chemical Biology*.

Many of these amino acids on the protein surface have reactive functional groups that will react with a substrate, even if the substrate is designed to target a different site. For example, cysteine has a thiol side chain. The thiol is highly reactive, which is helpful for many bodily processes, but makes selective chemical modification in the presence of a free cysteine difficult.

One site of interest for protein modification is the N-terminus part of the protein. This free amine is able to undergo various reactions, such as

acylation. However, most reactions involving the N-terminus are multi-step and are often specific to certain N-terminal amino acids. MacDonald et al.'s simple, one-step reaction expands on this work by selectively modifying the N-termini of a variety of proteins.

Their first step was to identify the right substrate to react with the free amine. Prior studies had shown that several types of aldehydes react with the N-termini to form 1:1 condensation products. Aryl aldehydes, specific derivatives of pyridinecarboxaldehydes, offer better site specificity because they form a cyclic imidazolidinone with the N-terminal amine and the neighboring amino acid. While a series of aryl aldehydes were tested as potential candidates for the modification of the N-termini of angiotensin I, 2-pyridinecarboxaldehyde (2PCA) offered the best yields under mild conditions. Further studies verified that 2PCA selectively reacted with the N-terminus of angiotensin I and that neighboring amino acids had little effect on reactivity. Further studies using these reaction conditions revealed that all possible N-terminal amino acids showed excellent yields, with proline and glycine as the least reactive.

The next step was to optimize the reaction conditions to make this an easily adaptable bioconjugation reaction. MacDonald et al. used RNase A as a model protein substrate, and included additives to the reaction mixture to replicate the chemistry of the protein surface. They found that RNase A was selectively modified at the N-terminus with optimal conditions at 10 mM 2PCA in 50 mM phosphate buffer at pH 7.5 and 37^{o}C for 16 hours. Protein concentration played little role in the success of the reaction, and the reaction tolerated a variety of buffers without interacting with the additives.

After optimizing the 2PCA reaction, the next step was to modify 2PCA with functional groups that are of interest for bioconjugation reactions. Other studies have shown that an acylation reaction with 2PCA and N-

hydroxysuccinimide (NHS) esters have worked well as a straight-forward way to modify 2PCA. MacDonald et al. were able to make protein-PEG bioconjugates using 2PCA-PEG. Characterization studies showed no side products due to non-site-specific reactions.

Importantly, this reaction worked well for a variety of commercially available proteins, including those with free cysteines. The authors tested their procedure with aldolase, a homotetramer with eight free cysteines per subunit. They were able to successfully modify the N-terminal group as well as selectively modify two of the eight free cysteines. They were also able to successfully modify 2PCA using a variety of substrates that might be of interest for protein modification.

The next question is whether the secondary and tertiary structures affect the 2PCA modification of the N-terminal group. Since a key part of the reaction is the formation of the cyclic imidazolidinone, steric hindrance from the three dimensional conformation of the protein may affect reactivity. They compared the reactivity of lysozyme and its N-terminal peptide analog. They found that the peptide was more reactive than the intact protein, confirming that access to the N-terminal group is an important factor in reactivity.

Finally, this procedure was used to modify estrogen receptor alpha (ERα'), a receptor that binds selectively to estrogen. This is of interest because of recent concerns over drinking water contaminated with certain hormone disruptors. ERα' was modified with 2PCA-biotin using the reported procedure, producing the bioconjugated product in 80% yield. The biotinylated ERα' was immobilized on a streptavidin resin and loaded with fluorescent ethinyl estradiol. In the presence of ethinyl estradiol, the fluorescent reporter is released from the binding site and detected by fluorescence spectroscopy. This demonstrates that the ERα' maintained its functionality even after modification, and it demonstrates a relatively simple method that might be used for detecting hormone

disruptors in drinking water.

Overall, this method is a simple, mild reaction that is adaptable to a diverse set of proteins. Furthermore, it works with native proteins without the need for genetic modification, does not seem to compromise protein functionality, and is site-specific even in the presence of highly reactive amino acids.

More information: "One step site-specific modification of native proteins with 2-pyridinecarboxyaldehyde" *Nature Chemistry Biology* published online: 30 march 2015. DOI: 10.1038/nchembio.1792