CHAPTER VI

Concluding Remarks and Future Work

SUMMARY OF THESIS WORK

All of the projects presented in this thesis involve the use of N-myristoyl transferase (NMT) to achieve selective and site-specific protein labeling in bacteria. Both *in vitro* and in live cells, NMT catalyzes the transfer of myristic acid, a fatty acid, to the N-terminus of its substrate proteins. While NMT plays important roles in eukaryotes, such as regulating signal transduction via myristoylation of its substrate proteins and enabling the infectivity of HIV and other viruses,[1,2] we examined NMT from the perspective of protein engineering. Specifically, we sought to exploit the tolerance of NMT toward reactive analogs of myristic acid and the ability of NMT to label engineered proteins displaying a recognition sequence derived from a natural substrate. Additional strengths that NMT naturally possesses are its specificity for the protein N-terminus, an attractive site for subsequent protein conjugation,[3] and its orthogonality toward endogenous bacterial proteins.[4] We took advantage of all of these features to develop a novel site-specific protein labeling system, and we evaluated our NMT-based system for different applications.

First, we constructed a bacterial model system for NMT-mediated protein functionalization, which required NMT itself, a test protein, and a reactive fatty acid tolerated by NMT. For these initial studies, summarized in Chapter II, we prepared two GFP-based substrate proteins, yARF-GFP and Fyn-GFP, named for the corresponding known NMT substrates, yARF and Fyn. In addition, we elected to incorporate 12-azidododecanoic acid (12-ADA), which we synthesized and purified, in place of myristic acid; we were drawn to the azide moiety because it can participate in three different bioorthogonal reactions.[5] Each GFP-based substrate protein was co-expressed

with NMT in *E. coli* in the presence of 12-ADA. Purification of each substrate protein and subsequent mass spectrometry experiments confirmed that NMT transfers 12-ADA to the protein N-terminus in quantitative yields. Treatment of lysate samples containing either engineered substrate protein with an azide-reactive fluorescent dye showed that NMT labeled only the substrate protein within the complex cellular environment of bacteria. The work presented in Chapter II constitutes the first example of NMT-mediated labeling of an engineered substrate protein with an analog of myristic acid.

As described in Chapter III, the methods developed for the GFP-based model system were applied to two proteins, calcineurin (CaN) and calmodulin (CaM), that are involved in learning and memory formation in mammals. CaN is naturally myristoylated; successful expression of CaN in bacteria actually requires co-expression with NMT in the presence of myristic acid.[6] We found that substitution of 12-ADA for myristic acid had no impact on the activity of CaN, as measured in a phosphatase activity assay. CaM is not naturally myristoylated, so we created a family of engineered CaM constructs and examined the impact that engineering and labeling had on the activity of each new protein. One construct, hCaNB-CaM, retained wild-type levels of activity and was carried forward for surface coupling work. Experiments similar to those described in Chapter II confirmed that NMT-mediated labeling of the CaN and CaM constructs was quantitative and selective.

Chapter IV details our work with protein microarrays. The yARF-GFP, CaN, and hCaNB-CaM proteins were each labeled with 12-ADA by NMT. Lysate samples containing each labeled substrate protein were incubated with cyclooctyne-spotted glass slides, and protein deposition was detected with the LFIRE (Label-Free Internal

Reflection Ellipsometry) instrument as a change in height at the slide surface. In this context, the orthogonality of NMT toward bacterial proteins is especially useful, as it enables surface immobilization of 12-ADA-labeled proteins without prior purification. To gain a better understanding of the kinetics of the azide-cyclooctyne reaction versus background reactions, we also measured the LFIRE signal over time. We found that significant surface capture of our 12-ADA-labeled proteins occurred within only 15 minutes, while the background signal remained relatively low. Furthermore, by comparing the percent of lysate protein constituted by CaN or hCaNB-CaM with the percent of coupled protein determined to be CaN or hCaNB-CaM, we calculated enrichment factors of 26 and ten for CaN and hCaNB-CaM, respectively. The methods we developed for our surface-coupling experiments comprise a strong foundation for completing high-throughput biochemical measurements with 12-ADA-labeled proteins captured directly out of lysate in a microarray format.

In the final section, Chapter V, we summarize our progress toward using NMT-mediated protein labeling to probe bacterial protein organization. Recent research has demonstrated that prokaryotes orchestrate the expression and movement of their proteins with greater sophistication than previously thought.[7,8] We believe that NMT would be an immensely useful tool for studying these phenomena, as NMT can functionalize a single predetermined substrate protein in bacteria for subsequent reaction with a dye or a probe. To that end, we identified an azide fatty acid, 7-azidoheptanoic acid, that is reactive, transferred by NMT to substrate proteins, and readily washed out of cells in its free form; in contrast, initial imaging experiments with 12-ADA showed that it is not removed from cells by standard wash protocols. We also prepared constructs encoding the bacterial

proteins PyrG and MreB modified with the yARF recognition sequence; both PyrG and MreB assemble into complex structures in cells and exhibit interesting localization patterns. Both yARF-PyrG and yARF-MreB expressed well, and the latter was robustly labeled with 7-azidoheptanoic acid or 12-ADA within 30 minutes. The tools and materials prepared for these experiments constitute a strong start toward the use of NMT-mediated protein labeling for *in vivo* imaging of bacterial proteins of interest.

In summary, the chapters comprising this thesis provide versatile methods and a strong case for the use of NMT in research projects involving site-specific functionalization of proteins. Potential applications include the conjugation of proteins to polymers for therapeutic ends, to surfaces for diagnostic microarrays, or to fluorophores for imaging studies. The N-terminus is well-suited for the covalent attachment of proteins to reactive partners because it is often surface-accessible, even in the folded state of a protein. A number of chemoenzymatic protein labeling techniques have been developed in the past decade, each with its own benefits and drawbacks.[9] The advantages of using NMT include the wide range of reactive myristic acid analogs that may serve as the fatty acid substrate, the small size of the recognition sequence required to achieve labeling of non-natural substrate proteins, and the orthogonality of NMT toward bacterial proteins. We envision many exciting possibilities for future research involving NMT-mediated protein labeling.

FURTHER STUDIES

Activity Measurements of Surface-Immobilized CaN and hCaNB-CaM

The larger goal of the work described in Chapter IV is the preparation of protein microarrays for high-throughput protein characterization studies, with an initial focus on measuring the activity of surface-bound CaN and hCaNB-CaM. Various methods are available for measuring CaN and CaM activity levels; as described in Chapter III, we employed an assay involving colorimetric detection of free phosphate resulting from Ca^{2+}/CaM-dependent dephosphorylation of a phosphopeptide substrate by CaN. This approach could be adapted to measure free phosphate generated by surface-bound proteins, rather than proteins in solution, perhaps by making use of suitably derivatized 96-well plates. Other colorimetric assays have also been developed, such as dephosphorylation by CaN of *para*-nitrophenyl phosphate (pNPP).[10] Alternatively, CaN or hCaNB-CaM could be immobilized on Biacor chips, enabling precise measurements of binding events.[11] These applications and others will be explored in the future by my collaborator, Dr. Tamara Kinzer-Ursem, in her lab at Purdue University.

Protein-Specific Imaging Studies in Bacteria

As described in Chapter V, we have developed nearly all of the necessary components for selective *in vivo* labeling and visualization of yARF-MreB. Dye-labeling experiments with lysates confirmed that bacterial co-expression of NMT and yARF-MreB in the presence of 7-azidoheptanoic acid results in labeling of only yARF-MreB; dye-labeling experiments with intact live cells indicated that the use of 7-azidoheptanoic acid yields no fluorescence signal above background. Thus, the next step is to dye-label

intact cells with a membrane-permeable and cytocompatible dye that reacts specifically with azides, such as cyclooctyne-coumarin[12] or cyclooctyne-BODIPY,[13] and conduct confocal imaging studies to elucidate where and how yARF-MreB is localized. Alternatively, other imaging methods with enhanced spatial resolution, such as electron cryotomography (ECT), could be utilized with suitable probes.[14,15] Finally, to gain a more accurate understanding of how yARF-MreB behaves in the cell, it would be advisable to replace the gene encoding MreB in the bacterial genome with the gene encoding yARF-MreB. This step would ensure that yARF-MreB is under the control of native promoters, so that its expression is turned "on" and "off" appropriately. Following the cloning methods described in Chapters II, III, and V, more constructs could be readily prepared that encode engineered bacterial proteins for labeling by NMT and subsequent visualization.

Controlled Conjugation and Release of N-Terminally Labeled Proteins from Hydrogels

During the course of this thesis, the development of functional biomaterials benefitting from NMT-mediated protein labeling had not been explored. However, work is currently underway to build upon the model system described in Chapter II to decorate hydrogels with site-specifically labeled proteins. In this context, the orthogonality of NMT toward bacterial systems is again beneficial: hydrogels displaying cyclooctyne or alkyne sites can simply be incubated with lysate containing a 12-ADA-labeled protein of interest for attachment of the labeled protein to the material. This project, led by Cole DeForest, a post-doctoral fellow in the Tirrell Lab, holds a great deal of potential.

Beyond the further studies described here, we are also excited by the idea of NMT-mediated protein labeling playing a role in entirely new applications. Moreover, while all of the projects described in this thesis took advantage of azide–alkyne reactions, other bioorthogonal chemistries could be explored, given the tolerance shown by NMT toward a wide variety of reactive fatty acid analogs. Similarly, dozens of recognition sequences have been identified, each possessing different steric and electrostatic characteristics depending on the residues comprising the sequence. Finally, nearly any protein can be engineered to display an NMT recognition sequence, with minimal perturbation of protein structure and function. The inherent characteristics of NMT, and its fascinating balance of specificity, selectivity, and promiscuity, render it a very powerful tool for protein-labeling studies. We hope that the reagents, methods, and results presented in this thesis for NMT-mediated protein labeling will be of great use in future biomedical research.

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