ARTICLE

Attempts towards Unambiguously Assigning ^{13}C-Dimethylamine NMR Resonances

Kevin J. Roberson and Megan A. Macnaughtan*

All Res. J. Chem., **2013**, 4, 10-16

All your results are good results!

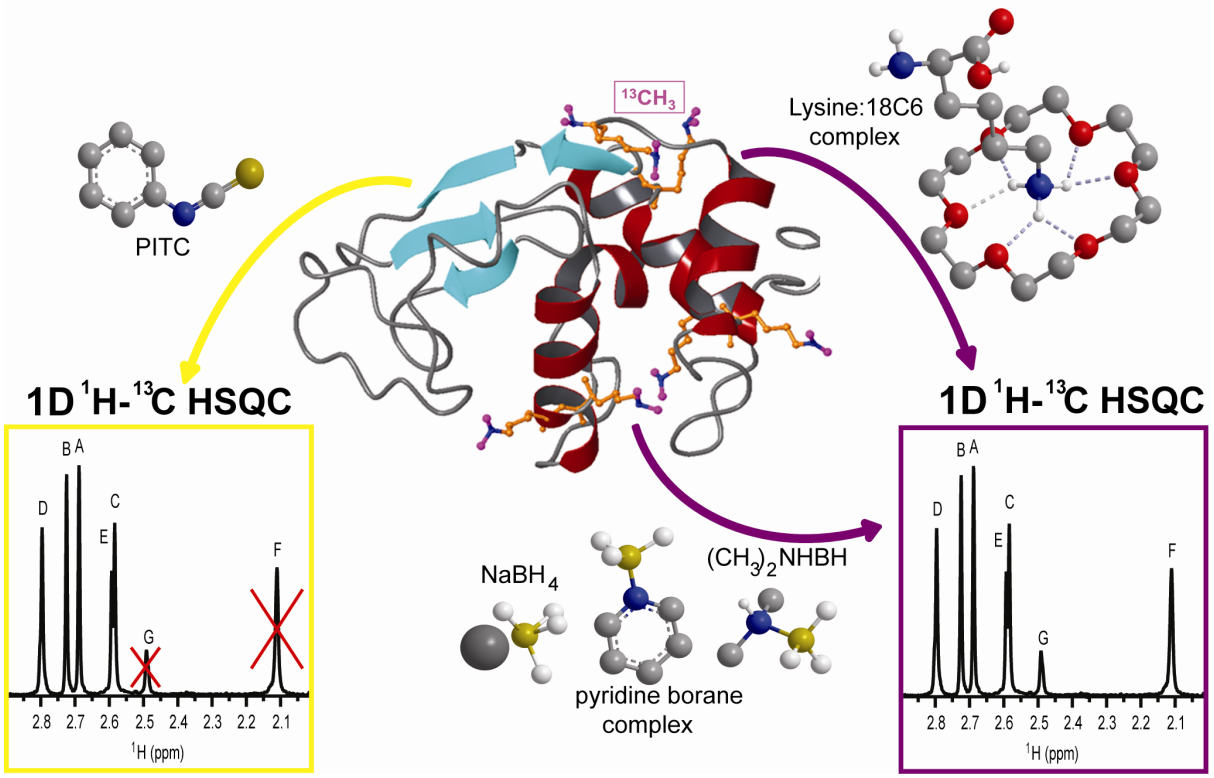
Issue 1, Vol 4, 2013, 10-16

Attempts towards Unambiguously Assigning ^{13}C-Dimethylamine NMR Resonances

Kevin J. Roberson and Megan A. Macnaughtan*

Louisiana State University, Department of Chemistry, Baton Rouge, LA 70803. Email: macnau@lsu.edu

Graphical Abstract



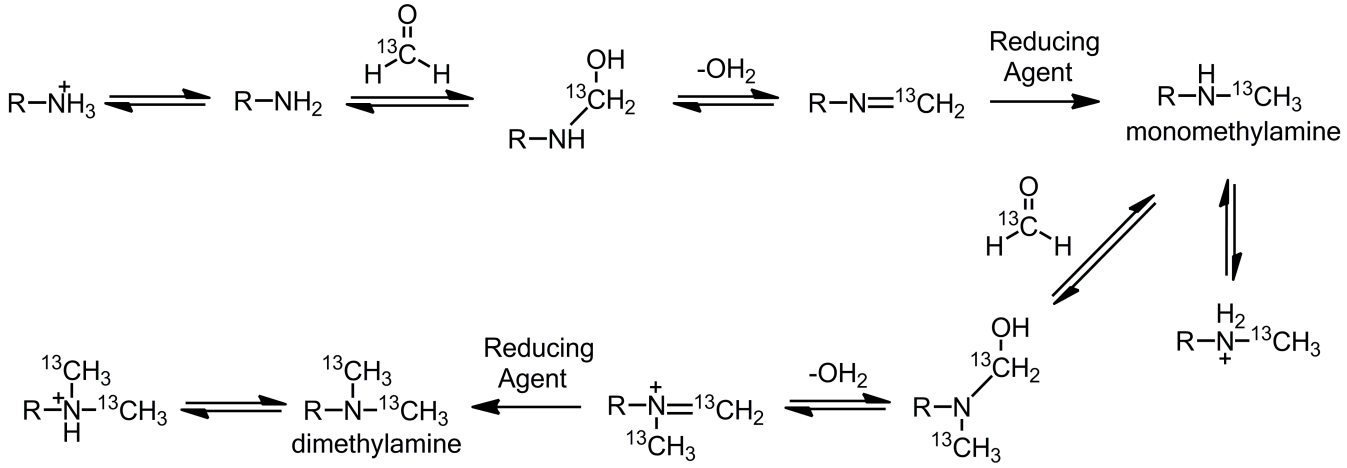
Abstract: Degenerate levels of ^{13}C-incorporation have been the biggest obstacle for mass spectrometry-assisted assignment of ^{13}C-dimethylamine resonances in nuclear magnetic resonance spectroscopy (NMR). Three methods are shown here to break the degeneracy in ^{13}C-labeling of lysozyme. Reductive methylation of lysozyme in the presence of 18-crown-6-ether is shown to hinder methylation but not in a selective manner. The use of multiple reducing agents, ranging in strength and hydrophobicity, proved to alter reaction rates in hydrophobic areas but labeling was still degenerate. The development of a non-destructive Edman degradation to remove the problematic *N*-terminal lysine for the assignment of NMR resonances associated with both α- and ε-dimethylamines proved elusive.

Keywords: Reductive methylation, protein, 18-crown-6-ether, Edman degradation, reducing agent, lysine

Introduction

As a tool for protein structure and dynamics studies, nuclear magnetic resonance spectroscopy typically requires stable magnetic isotope enrichment to improve signal intensity and allow assignment.[1] Complete isotopic labeling is most commonly achieved through overexpression in *Escherichia coli* with ^{15}N-ammonium chloride and ^{13}C-glucose.[2,3] Because many human proteins require eukaryotic hosts for proper folding and post-translational modifications, metabolic labeling is not always viable. An alternative approach is sparse isotopic labeling using protein-chemical modification with highly selective reactions for specific amino acids.[4] ^{13}C-methyl tagging via reductive methylation has been successful in both NMR and X-ray crystallographic

protein studies. Under non-denaturing conditions, the *N*-terminal α-NH_2 and the lysine side chain ε-NH_2 groups are selectively methylated in the presence of formaldehyde and a reducing agent (Scheme 1). The reaction produces dimethylamino groups because the monomethylamine readily reacts due to a higher pK_a than the non-methylated primary amine.[5,6] By using ^{13}C-formaldehyde, ^{13}C-labels are incorporated into the protein in the form of ^{13}C-methyls.[7] Once incorporated, protein structure and dynamics are usually not perturbed.[8] The ^{13}C-methyls can be used as probes in NMR experiments to study protein-protein interactions and as sites to collect distance constraints using paramagnetic perturbations.[9,10]



Scheme 1: The reductive methylation reaction: In the presence of formaldehyde and a reducing agent, the primary amine is reductively methylated to produce monomethylamine. In the presence of excess formaldehyde, the monomethylamine undergoes a second reductive methylation to produce dimethylamine.

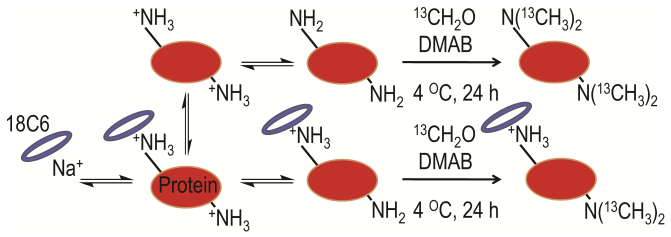
Since its introduction as a means for protein modification by Means and Feeney in 1968,[5] reductive methylation has been used to incorporate probes of structural and dynamic properties.[9-16] When the ^{13}C-methyl groups are used to study proteins with NMR, the utility of the labeling method is limited by difficulties in assigning the ^{13}C-methyl resonances with their corresponding lysine residues. Past assignment approaches have relied on a small number of methylation sites,[14,17] known structural properties,[7,11,15,18-20] or genetic modifications.[21] One strategies used matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) to identify partially-methylated lysines of tryptic peptides of human MIP-1α by correlating the disappearance of unmodified peptide masses in MS with the appearance of mono- and dimethylamine signals in 2D ^{1}H-^{13}C HSQC-NOESY NMR spectra.[17] In 2005, we presented an improved method for assigning dimethylamine resonances in 2D ^{1}H-^{13}C HSQC NMR spectra that requires no prior knowledge of the protein except the amino acid sequence.[22] The mass spectrometry-assisted assignment method uses isotopic ratio measurements. Using limiting ^{13}C-formaldehye, this technique takes advantage of slightly differing reaction rates at each site, based on pK_a values and steric accessibility, to distinguish sites in NMR and MS data. Data presented on this procedure using lysozyme showed two inherent problems with the strategy: 1) degenerate levels of ^{13}C-incorporation and 2) no MS method to independently measure the ^{13}C-incorporation of the α- and ε-dimethylamines on Lys1.[22]

To overcome the degeneracy in labeling lysozyme with the reductive methylation reaction, we investigated two methods: 1) reductive methylation in the presence of 18-crown-6-ether (18C6) and 2) reductive methylation using multiple reducing agents. To assign the α- and ε-dimethylamine resonances of the *N*-terminal lysine residue of lysozyme, we explored a non-destructive Edman degradation method.

Results and Discussion

Reductive Methylation in the Presence of 18-crown-6-ether

18C6 is known for its ability to form non-covalent complexes to metal cations and protonated primary amines in both solution and gas phase,[23] hence its extensive use in peptide synthesis[23-26] and purification.[27-30] The 18C6 and protonated primary amine complex forms through a combination of three hydrogen bonds and ion-dipole interactions. It is through this chemistry we postulated the concept shown in Scheme 2 to break the degenerate levels of ^{13}C-incorporation for reductively methylated lysozyme. In solution, the lysine side chain amine exists in equilibrium between the protonated primary amine and the neutral primary amine. 18C6 complexes with the protonated form, which is not the reactive species, and should hinder methylation at the site. Binding affinities of 18C6 should be slightly different at each site based on steric accessibility and influences from surrounding residues, altering the relative reductive methylation rates.



Scheme 2: Reductive methylation in the presence of 18C6.

Initial methylation attempts in the presence of 18C6 were performed by varying the ratio of reactive amine concentration to 18C6. Results showed that the competitive binding of the buffer-counter ion inhibited binding of 18C6 to the reactive amine. Potassium ion binds better to 18C6 than the sodium ion, which binds slightly better than the ammonium ion, while the lithium ion does not bind as well.[31,32] For this reason, the potassium buffer was avoided and the sodium and lithium buffers were tested. Lysozyme was reductively methylated in the presence of a sodium phosphate buffer (20 mM Na^+) at ratios of 2:1 and 1:1 (moles of Na^+: moles of 18C6). In each experiment, lysozyme was reacted in the presence of 18C6 at pH 7.5. As shown in Figure 1, 18C6 had no effect on the methylation of lysozyme at these molar ratios, presumably because of the poor fit of the protein ammonium ion in the 18C6 cavity or the stronger binding of 18C6 to the sodium ions. Both possibilities were tested by methylating lysozyme in the presence of excess 18C6 at ratios of 2:3 and 2:5 (counter ion: 18C6), where the counter ion is either sodium or lithium (Figure 2). In each of these reactions, lysozyme was reacted in the presence of 18C6 at pH 7.5. When in excess of the counter ion, 18C6 hinders reductive methylation of lysozyme; however, the effect is equivalent across each reactive site and did not solve the degenerate labeling problem, indicating that the affinity of the protein ammonium ions for 18C6 are not significantly different. Switching the counter ion from sodium to lithium showed no significant differences in methylation, indicating that excess 18C6 is more important than counter-ion selection.

Reductive Methylation Using Multiple Reducing Agents

In 1995, Means and Feeney reviewed the reductive methylation reaction and its efficiency as it related to different reducing agents.[33] The strength of the reducing agent was found to be inversely related to the efficiency of methylation. Here, we attempted to take advantage of the

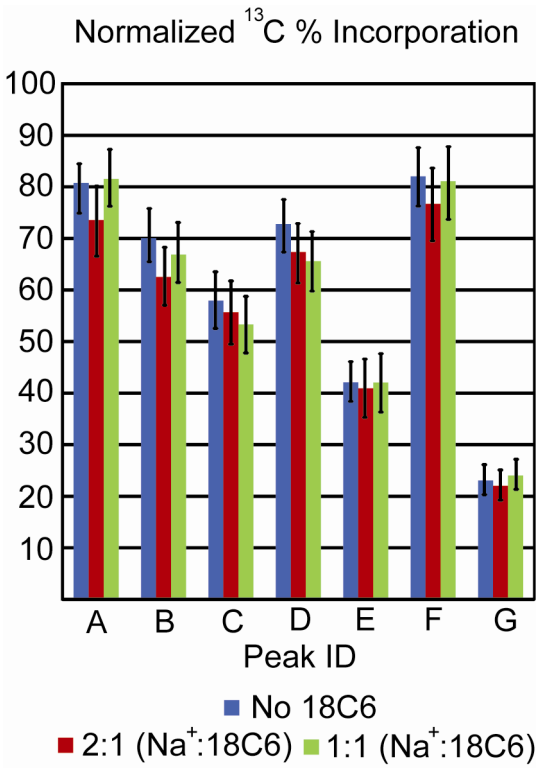


Figure 1: Normalized NMR ^{13}C percent incorporation of each reaction site of partially-labeled lysozyme at 1:5 ratio (moles of reactive amine: moles of formaldehyde) in the presence of 18C6 and sodium ion.

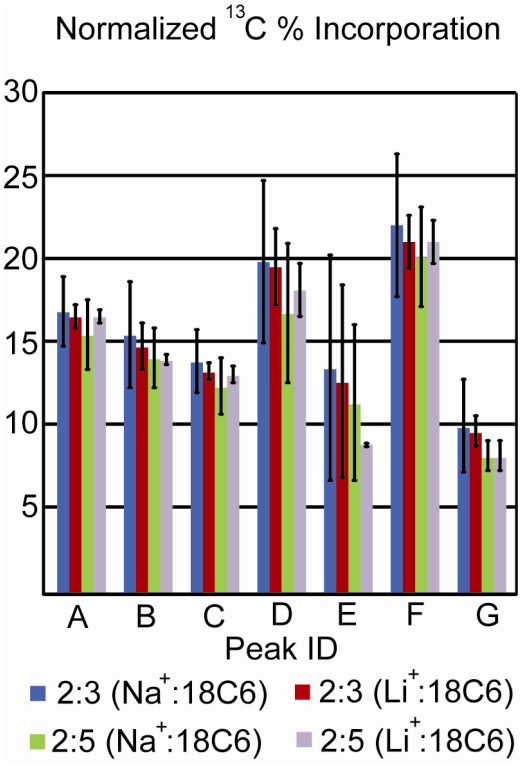


Figure 2: Normalized NMR ^{13}C percent incorporation of each reaction site of partially-labeled lysozyme at 1:5 ratio (reactive amine: formaldehyde) in the presence of excess 18C6 over sodium and lithium ions.

inefficiency of the stronger reducing agents, hypothesizing that the kinetic mode of action will be determined by the physical properties of the reducing agent and reactive site. For instance, a reducing agent with a hydrophobic structure, like pyridine borane, would likely reduce a Schiff base in a hydrophobic pocket faster than a hydrophilic reducing agent and, in turn, break the degeneracy in the ^{13}C-incorporation across the reactive sites. Figures 3a-e are structural representations of the hydrophobic regions of lysozyme (yellow) with each lysine residue (orange) labeled. Reducing agents used in these experiments, in order of increasing strength, were dimethylamine borane complex (DMAB), pyridine borane complex, and sodium borohydride (Figure 3f).

Lysozyme was reductively methylated with a sub-stoichiometric amount of ^{13}C-formaldehyde in the presence of each reducing agent for partial labeling, and then methylated with excess natural abundance formaldehyde in the presence of dimethylamine borane complex to complete methylation. Reactions were performed at both 4 and 25°C and yielded the same results (data shown for 4°C). Changing the reducing agent had no significant effect on the degenerate labeling between peaks A and D, as shown in Figure 4. While the different reducing agents produced slightly different levels of ^{13}C, most of the rates were degenerate within error.

Interestingly, peaks D and F were reductively methylated at a faster rate with the small, hydrophobic DMAB than the hydrophilic $NaBH_4$. Previous studies have assigned Peak D as K97[19,34] and peak F as the *N*-terminal α-NH_2,[19,34-36] which were found to be sandwiched between hydrophobic regions (Figure 3). These findings confirm our hypothesis that the hydrophobic DMAB would reduce a Schiff base in a hydrophobic region faster than a more hydrophilic reducing agent.

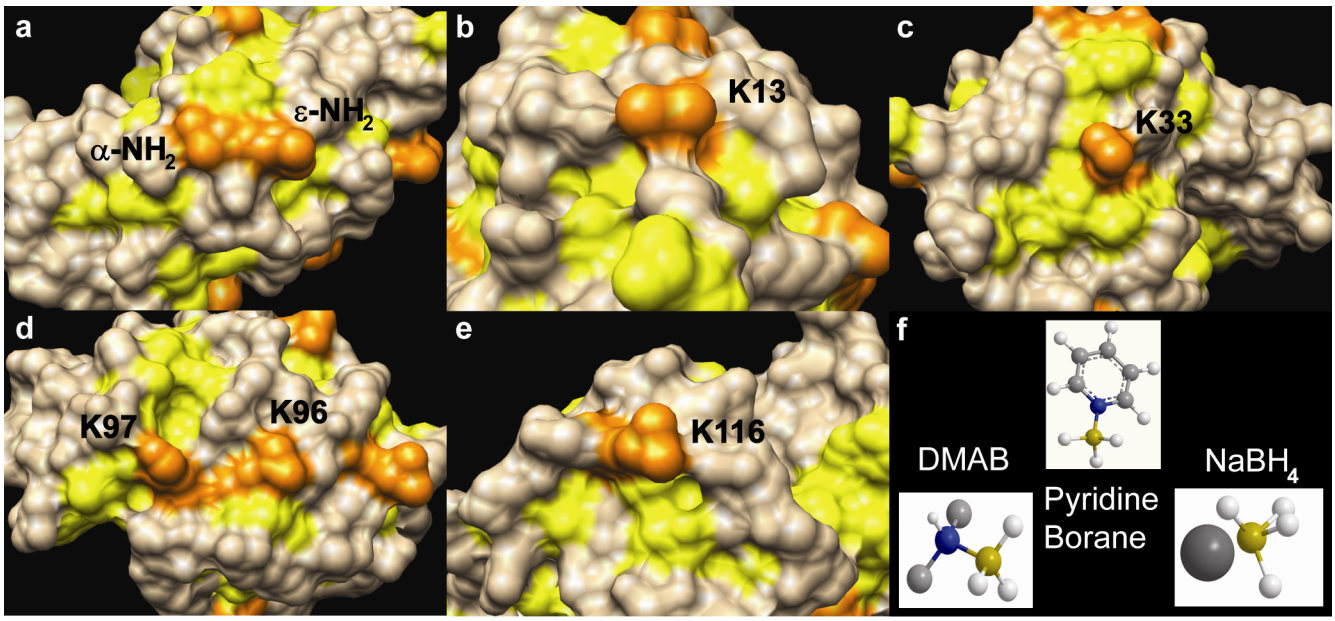


Figure 3: (a-e) Using the crystal structure of reductively methylated lysozyme by Rypniewski, et. al (PDB ID: 132L),[8] the dimethylamino groups (orange) and hydrophobic residues (yellow) are highlighted. (f) The structures of the borane reducing agents.

Non-destructive Edman Degradation

Since its introduction in 1950, Edman degradation has been used to determine the amino acid sequence of proteins[37,38] until recent advances in mass spectrometry. Phenylisothiocyanate (PITC) is reacted with the *N*-terminal amine under mildly alkaline conditions to form a cyclical phenylthiocarbamoyl derivative. When treated with acid, the derivatized terminal amino acid is cleaved. After extraction, it is further treated with acid to form the more stable phenylthiohydantoin (PTH)-amino acid derivative. During this process, the protein is sequentially cleaved to determine the amino acid sequence. For our purpose, we were not necessarily interested in the cleaved PTH-amino acid derivative, but rather the remaining protein for structural studies; therefore it was important that the truncated protein remain folded. Our plan was to use Edman degradation to remove the *N*-terminal lysine residue of lysozyme (Lys1) to

assign the NMR peaks belonging to the α- and ε-dimethylamine of Lys1. Upon NMR analysis, the Lys1 ε-

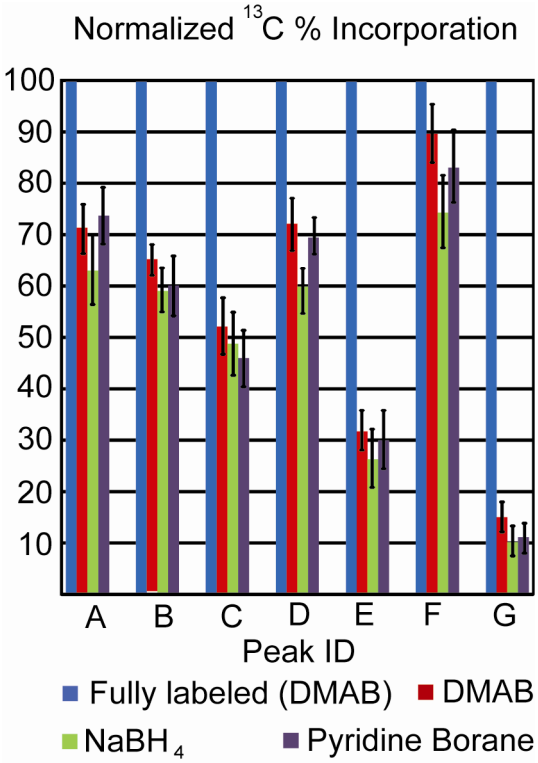


Figure 4: ^{13}C percent incorporation of partially ^{13}C-reductively methylated lysozyme with different reducing agents normalized to fully ^{13}C-methylated lysozyme with DMAB.

dimethylamine peak will be absent and a new *N*-terminal α-dimethylamine resonance will be present allowing the assignment of both the α- and ε-dimethylamine resonances of Lys1.

PITC was coupled to the *N*-terminus of lysozyme, the sample was freeze-dried, reconstituted, and analyzed with ^{1}H NMR to verify the tertiary structure. The broad range of resonances in the ^{1}H NMR spectrum (not shown) confirmed that the PTH-protein was folded. Difficulty followed in attempts to cleave the derivatized amino acid from the protein. Initially, lysozyme was dissolved in pure trifluoroacetic acid (TFA) or HCl for cleavage. The acid was then exchanged for acetonitrile before the buffer was exchanged to phosphate to prevent further degradation of the protein by acid hydrolysis, since cleavage is sensitive to aqueous conditions. NMR analysis showed that the protein was unfolded and prompted another approach to preserve the protein's tertiary structure.

Since lysozyme unfolded after cleaving with pure TFA and HCl, we investigated the use of other organic solvents in which lysozyme was soluble and not destructive to the protein structure. In a study conducted by Chin *et al.*, lysozyme was reported to be soluble in many organic solvents at concentrations greater than 10mg/mL.[39] Here, we used methanol, dimethyl sulfoxide (DMSO), glycerol, and formamide as solvents for lysozyme before adding TFA (final concentration of 75%) for cleavage. After cleavage, NMR studies showed that lysozyme unfolded in all solvents when treated with TFA. Attempts to refold the protein were also unsuccessful. In summary, all experiments performed under acidic conditions resulted in unfolded lysozyme. We also attempted cleavage studies in basic conditions using a similar co-solvent system to that of the coupling reaction previously described by Barrett and Penglis.[40] During the cleavage step at 75°C, the protein precipitated. Precipitation was avoided when cleavage was performed at 50°C, but NMR showed the protein unfolded. Moreover, cleavage studies were also unsuccessful using the same conditions as the coupling reaction, with a mixture of pyridine, triethylamine, and water (1.2:15:10). To date, protein stability in association with the cleavage of the phenylthiocarbamoyl amino acid derivative has yet to be resolved.

Conclusions

Here we presented two theoretically sound methods to eradicate the degeneracy of ^{13}C-labeling via reductive methylation of lysozyme and one method to assign the α- and ε-dimethylamine of Lys1. The reductive methylation of lysozyme in the presence of 18C6 was found to hinder the extent of methylation but was not selective. Although a series of reducing agents were successful in reductively methylating lysozyme, they also did not produce the desired selectivity. Non-destructive Edman degradation was promising, but unsuccessful in preserving the protein's tertiary structure.

Experimental

Reductive methylation in the presence of 18C6

Fully ^{13}C-labeled control sample: To an aqueous solution of lysozyme (2.5mg, 5mg/mL), DMAB (6.13µL, 1M) was added, followed by ^{13}C-formaldehyde (12.25µL, 1M). The reaction mixture was shaken at 4°C for 2 hours. A second aliquot of DMAB and ^{13}C-formaldehyde was added, and the mixture was shaken at 4°C for an additional 2 hours. DMAB (3.06µL, 1M) was added, and the mixture was shaken at 4°C overnight for a total reaction time of 24 hours.

Partial ^{13}C-labeling (2:1) and (1:1): To an aqueous solution of lysozyme (5mg/mL), an aliquot of 18C6 (0.66mg, 5µmol or 1.32mg, 10µmol) was added for final concentrations of 5 and 10mM respectively, followed by DMAB (1.53µL, 1M), then ^{13}C-formaldehyde (3.06µL, 1M). The mixture was

shaken at 4°C for 2 hours. A second aliquot of DMAB and ^{13}C-formaldehyde were added, and the mixture was shaken at 4°C for an additional 2 hours. DMAB (0.77µL, 1M) was then added, and the mixture was shaken at 4°C overnight for a total of 24 hours.

For reactions at ratios 2:3 and 2:5, an aliquot of 18C6 (4.0 mg, 15µmol or 6.6mg, 25µmol) was added for final concentrations of 30 and 50mM, respectively.

Excess natural abundance formaldehyde to complete methylation: To an aqueous solution of lysozyme (5mg/mL), DMAB (6.13µL of 1M) was added, followed by formaldehyde (12.25µL of 1M). The mixture was shaken at 4°C for 2 hours. A second aliquot of DMAB and formaldehyde were added, and the mixture was shaken at 4°C for an additional 2 hours. DMAB (3.06µL, 1M) was then added, and mixture was shaken at 4°C overnight for a total of 24 hours.

Reductive methylation using multiple reducing agents

The procedures for fully and partially ^{13}C-labeling and excess natural abundance formaldehyde described above were used without 18C6 and with various reducing agents. One fully ^{13}C-labeled control sample with DMAB and partially ^{13}C-labeled samples with each reducing agent were prepared. All reducing agent aliquots were from 1M stock solutions except sodium borohydride, which was added as a solid in small portions over time due to its high reactivity.

Preparation for NMR

The protein samples were exchanged into a D_2O, 50 mM sodium borate buffer at pH 8.5 using an Amicon Ultra 4mL centrifugal filter with a 3kDa molecular weight cutoff.

NMR

All 1D ^{1}H - ^{13}C heteronuclear single-quantum coherence (HSQC) NMR spectra were collected using a 700 MHz Varian spectrometer equipped with a 5 mm-HCN-5922 probe. 1,2-dichloroethane-$^{13}C_2$ (26 mM in D_2O) was used as an external reference via coaxial insert. The protein concentrations were 50 µM. Experiments were acquired at 25°C. All experiments were acquired using a relaxation delay of 5s, 256 scans, and a 4529Hz spectral width. The total acquisition time for each experiment was approximately 25 minutes. 1,2-dichloroethane-$^{13}C_2$ was used as a chemical shift reference instead of the traditional DSS.

The 1D ^{1}H-NMR of the PITC-lysozyme was acquired using a relaxation delay of 1.5s, 4 scans, and a 7022.5 spectral width. The spectra were referenced to DSS.

^{13}C percent incorporation calculation from NMR data

1D ^{1}H-^{13}C HSQC was performed on a fully ^{13}C-dimethylated (control) protein sample. The area under each dimethylamine peak in the spectrum was integrated and set to 100% ^{13}C-incorporation for each site. The areas of the corresponding peaks in the spectra for the partially ^{13}C-dimethylated samples were integrated. ^{13}C percent incorporation of the dimethylamine peaks of the partially ^{13}C-methylated samples was calculated as a fraction of the respective peak for the control protein sample.

Non-destructive Edman degradation

Coupling: Lysozyme (10mg, 0.680µmol) was dissolved in pyridine, triethylamine, and water (1.2:15:10) co-solvent. PITC (1.3mg, 9.55µmol) was added, and the reaction was stirred at 50°C for 30 minutes.

Cleavage:

(1): PTH-lysozyme was dissolved in 100% TFA or concentrated HCl and stirred for 30 minutes at room temperature.

(2): PTH-lysozyme was dissolved in an organic solvent. TFA was added to make a 75% solution and stirred for 30 minutes at room temperature.

(3): PTH-lysozyme was dissolved in triethylamine, acetic acid, and acetonitrile (7.5:3:5) and stirred at 50°C for 30 minutes.

Acknowledgments: This research was supported by Award Number R00RR024105 from the National Center For Research Resources, National Institute of Health.

Molecular graphics were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).[41]

References

1. Wuthrich, K. *NMR of Proteins and Nucleic Acids,* John Wiley and Sons: New York, **1986.**
2. Shimba, N., Yamada, N., Yokoyama, K. and Suzuki, E. *Anal. Biochem.,* **2002,** *301,* 123-127.
3. Palomares, L., Estrada-Moncada, S. and Ramírez, O. *Production of Recombinant Proteins*, Second; P. Balbás and A. Lorence, Humana Press: New York City, New York, USA, **2004**; 267, 15-51.
4. Lundblad, R. *Chemical Reagents for Protein Modification,* CRC Press: Boca Raton, FL, **1991.**

5. Means, G. and Feeney, R. *Biochemistry,* **1968,** *7,* 2192-2201.
6. Abraham, S., Hoheisel, S. and Gaponenko, V. *J Biomol. NMR,* **2008,** *42,* 143-148.
7. Jentoft, J. E., Jentoft, N., Gerken, T. A. and Dearborn, D. G. *J. Biol. Chem.,* **1979,** *254,* 4366-4370.
8. Rypniewski, W., Holden, H. and Rayment, I. *Biochemistry,* **1993,** *32,* 9851-9858.
9. Moore, G. R., Cox, M. C., Crowe, D., Osborne, M. J., Rosell, F. I., Bujons, J., Barker, P. D., Mauk, M. R. and Mauk, A. G. *Biochem. J.,* **1998,** *332,* 439-449.
10. Dick, L. R., Geraldes, C., Sherry, A. D., Gray, C. W. and Gray, D. M. *Biochemistry,* **1989,** *28,* 7896-7904.
11. Brown, L. and Bradbury, J. *Eur. J. Biochem.,* **1975,** *54,* 219-227.
12. Jentoft, J., Gerken, T., Jentoft, N. and Dearborn, D. *J. Biol. Chem.,* **1981,** *256,* 231-236.
13. Goux, W., Teherani, J. and Sherry, A. *Biophys. Chem.,* **1984,** *19,* 363-373.
14. Dick, L., Sherry, A., Newkirk, M. and Gray, D. *J. Biol. Chem.,* **1988,** *263,* 18864-18872.
15. Jentoft, J. E. *Methods Enzymol.,* **1991,** *203,* 261-295.
16. Sparks, D., Phillips, M. and Lundkatz, S. *J. Biol. Chem.,* **1992,** *267,* 25830-25838.
17. Ashfield, J., Meyers, T., Lowne, D., Varley, P., Arnold, J., Tan, P., Yang, J., Czaplewski, L., Dudgeon, T. and Fisher, J. *Protein Sci.,* **2000,** *9,* 2047-2053.
18. Sherry, A. and Teherani, J. *J. Biol. Chem.,* **1983,** *258,* 8663-8669.
19. Gerken, T., Jentoft, J., Jentoft, N. and Dearborn, D. *J. Biol. Chem.,* **1982,** *257,* 2894-2900.
20. Huque, M. and Vogel, H. *J. Protein Chem.,* **1993,** *12,* 695-707.
21. Zhang, M. and Vogel, H. *J. Biol. Chem.,* **1993,** *268,* 22420-22428.
22. Macnaughtan, M., Kane, A. and Prestegard, J. *J. Am. Chem. Soc.,* **2005,** *127,* 17626-17627.
23. Julian, R. R., May, J. A., Stoltz, B. M. and Beauchamp, J. L. *Int. J. Mass Spectrom.,* **2003,** *228,* 851-864.
24. Botti, P., Ball, H. L., Lucietto, P., Pinori, M., Rizzi, E. and Mascagni, P. *J. Pept. Sci.,* **1996,** *2,* 371-380.
25. Botti, P., Ball, H. L., Rizzi, E., Lucietto, P., Pinori, M. and Mascagni, P. *Tetrahedron,* **1995,** *51,* 5447-5458.
26. Hyde, C. B. and Mascagni, P. *Tetrahedron Lett.,* **1990,** *31,* 399-402.
27. Poll, D. J. and Harding, D. R. K. *J. Chromatogr.,* **1991,** *539,* 37-45.
28. Josic, D., Reutter, W. and Reusch, J. *J. Chromatogr.,* **1989,** *476,* 309-318.
29. Julian, R. R. and Beauchamp, J. L. *Int. J. Mass Spectrom.,* **2001,** *210,* 613-623.
30. Julian, R. R. and Beauchamp, J. L. *J. Am. Soc. Mass Spectrom.,* **2002,** *13,* 493-498.
31. Liou, C. C. and Brodbelt, J. S. *J. Am. Chem. Soc.,* **1992,** *114,* 6761-6764.
32. Maleknia, S. and Brodbelt, J. *J. Am. Chem. Soc.,* **1992,** *114,* 4295-4298.
33. Means, G. E. and Feeney, R. E. *Anal. Biochem.,* **1995,** *224,* 1-16.
34. Bradbury, J. H. and Brown, L. R. *Eur. J. Biochem.,* **1973,** *40,* 565-576.
35. Larda, S. T., Bokoch, M. P., Evanics, F. and Prosser, R. S. *J. Biomol. NMR,* **2012,** *54,* 199-209.
36. Roberson, K. J., Brady, P. N., Sweeney, M. M. and Macnaughtan, M. A. *J. Visualized Exp.,* **2013,** *"In Press",*
37. Edman, P. *Acta Chem. Scand.,* **1950,** *4,* 277-282.
38. Edman, P. *Acta Chem. Scand.,* **1950,** *4,* 283-293.
39. Chin, J. T., Wheeler, S. L. and Klibanov, A. M. *Biotechnol. Bioeng.,* **1994,** *44,* 140-145.
40. Barrett, G. C., Penglis, A. A. E., Penrose, A. J. and Wright, D. E. *Tetrahedron Lett.,* **1985,** *26,* 4375-4378.
41. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C. and Ferrin, T. E. *J. Comput. Chem.,* **2004,** *25,* 1605-1612.