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Mass Spectrometry Assisted Assignment of NMR Resonances in Reductively ^{13}C-Methylated Proteins

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Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for studying protein structure and dynamics, but in most cases, it relies on extensive incorporation of stable magnetic isotopes to aid in resolution and assignment. Typically, overexpression in a bacterial host is used to uniformly incorporate ^{13}C, ^{15}N, and ^{2}H into a protein. For eukaryotic proteins that are less amenable to bacterial host expression (membrane proteins or glycosylated proteins), labeling through overexpression in eukaryotic hosts can be difficult and expensive. An alternate approach is to isotopically label proteins post-expression via chemical methods. Chemical modification of proteins is an established field of science with highly selective reactions for certain amino acids.[1] In many cases, the products also mimic those produced by natural post-translational modifications. One protein modification that has already been used successfully with NMR and X-ray crystallography is reductive methylation of lysines. In principle, for NMR, the signals from ^{13}C methyls introduced in this way can be sensitive probes of protein−protein interactions and easily observable sites for collection of distance restraints from paramagnetic perturbations.[2] However, assignment of resonances is a prerequisite for these applications, and this has always been a problem. In this communication, we offer a solution to that problem.

Reductive methylation of proteins was introduced by Means and Feeney in 1968.[3] Under mild reaction conditions, the lysine ϵ-NH_2 and N-terminal α-NH_2 can be selectively methylated using ^{13}C-labeled formaldehyde and a reducing agent, such as dimethylamine borane, to produce the dimethylated amine. Since its introduction, it has been used a number of times to introduce probes of both dynamic and structural properties.[2,4,5] Assignment approaches have varied, but for the most part, these have relied on small numbers of sites, known structural properties of proteins, or extensive genetic modification.[6] In 2000, Fisher et al. used MALDI-TOF to identify partially methylated lysines of trypsin-digested human MIP-1α by correlating the disappearance of unmodified peptide masses in MS with the appearance of mono- and dimethylamine signals observed in a 2D $^{1}H-^{13}C$ NMR spectrum.[7] Here we present an improved method for assigning dimethyllysine and dimethylamine resonances in 2D $^{1}H-^{13}C$ NMR spectra without prior knowledge of the protein's structure or properties other than the amino acid sequence. This method builds on the previous use of mass spectrometry, but introduces isotope ratio measurements that make quantitation and more extensive assignments possible. The method relies on different rates of reductive methylation at each primary amine site in a protein; these are expected to vary with factors that are known to affect the rate of reductive methylation, such as individual pK_a's and steric accessibility of the various primary amines.[5,7]

Reductive methylation of hen egg white lysozyme with ^{13}C-formaldehyde was carried out following the procedure described by Rayment.[8] Dimethylamine borane complex at 20 mM was used as the reducing agent, and various amounts of ^{13}C-formaldehyde were used to partially reductively ^{13}C-methylate 100 μL aliquots containing 0.5 mg of lysozyme at pH 7.5 in phosphate buffer at 4 °C. Four ^{13}C-labeled samples were prepared with 0.12, 0.24, 0.49, and 1.22 μmoles of ^{13}C-formaldehyde to give molar ratios of ^{13}C-formaldehyde to primary amine of 0.5:1, 1:1, 2:1, and 5:1. These samples were subsequently reacted with excess natural abundance formaldehyde at 40 mM to complete the reductive methylation and produce homogeneously modified protein samples. The reaction was quenched with 100 mM ammonium sulfate, and the samples were washed into a similar buffer suitable for NMR. An additional sample was prepared using excess ^{13}C-formaldehyde and was used as a normalization reference for the NMR and MS data.

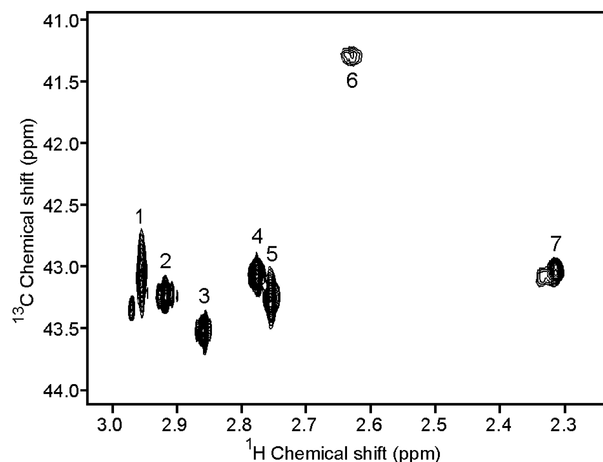

Figure 1. $^{1}H-^{13}C$ HSQC of ^{13}C-dimethylated lysozyme (120 μM) at pH 8.1 acquired on a 600 MHz spectrometer in approximately 5 min.

Two-dimensional $^{1}H-^{13}C$ heteronuclear single quantum correlation spectroscopy (HSQC) NMR and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) were used to analyze all reductively methylated lysozyme samples. An NMR spectrum of the fully ^{13}C-dimethylamine-labeled lysozyme sample is shown in Figure 1. The seven expected dimethylamine resonances are observed with high sensitivity and good resolution in the 41−43 ppm ^{13}C chemical shift range. As expected, the monomethylamine region around 32 ppm (not shown) did not show any resonances. Complete reductive methylation of the protein is essential for both quality NMR spectra and quantitative analysis of spectra. Incomplete methylation gives both dimethylamine and monomethylamine resonances. These are resolved in themselves; however, effects of differential methylation of proximate sites perturb chemical shifts, leading to poor quality spectra.[5]

Assignment of the dimethylamine NMR peaks was accomplished by correlating the amount of ^{13}C incorporation into each dimethylamine group for the four partially ^{13}C-labeled samples. In the NMR spectra, each peak was integrated and normalized to the reference ^{13}C-dimethylamine lysozyme sample. Table 1 lists the ^{13}C incor-

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Table 1. Normalized Peak Volumes from ^{1}H−^{13}C HSQC Spectra of the Four Partially ^{13}C-Labeled Samples[a]

NMR peak	0.5:1 sample	1:1 sample	2:1 sample	5:1 sample
1	9.9	18.0	33.7	71.4
2	8.8	12.8	25.3	61.1
3	11.6	17.3	32.0	70.3
4	5.2	7.5	15.4	38.6
5	8.8	11.9	26.1	60.0
6			10.7	30.2
7	19.9	21.5	34.7	78.3

[a] The molar ratio of ^{13}C-formaldehyde to primary amine used in the ^{13}C reductive methylation reaction is used to name each sample.

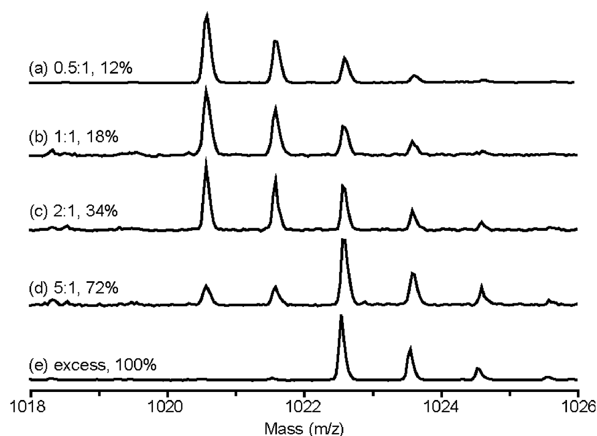

Figure 2. MS isotope profiles for the tryptic peptide containing a single dimethyllysine (Res. #6−14 CELAAAMKR) at various reaction stoichiometries. The reaction conditions in moles of ^{13}C-formaldehyde relative to the moles of reactive amines are listed along with the normalized percentages of ^{13}C incorporation calculated from the profiles.

poration percentage for each peak and each sample. As expected, the amount of ^{13}C incorporated at each site (lysine or N-terminal amine) increased with increasing moles of ^{13}C-formaldehyde used in the reaction mixture. More importantly, for most ratios, the extent of ^{13}C incorporation at each site differed, indicating that the individual chemical environments play a role in the reaction and the dimethylamine groups can be distinguished on this basis.

A MALDI-TOF/TOF MS (Applied Biosystems 4700 Proteomics Analyzer) was used with an α-cyano-4-hydroxycinnamic acid/acetonitrile matrix to measure levels of ^{13}C incorporation in specifically identified peptide sequences. A small aliquot from each sample was proteolyzed with trypsin (trypsin does not cleave efficiently at dimethyllysine, so primarily arginine sites were cleaved). Figure 2 shows the MALDI-TOF data from the peptide containing lysine 13 for every sample. The average mass in the profile shifts by 2 mass units as the amount of ^{13}C-formaldehyde used is increased; this limit corresponds to the final addition of 2 ^{13}C's in the methyl groups. For each peptide containing a dimethylamine group, the average mass was calculated at each reaction ratio. The reference ^{13}C-dimethylamine lysozyme sample was used to correct for natural abundance contributions at non-methyl sites and to normalize the data to give the percentages of ^{13}C incorporation listed in Table 2.

Assignment of the NMR peaks can be made by comparing the NMR and MS percentages of ^{13}C incorporation in Tables 1 and 2. While all reaction ratios can contribute to assignment, we illustrate this with data from the 5:1 reaction. The four peptides containing single lysines, including K13, K33, K97, and K116, are simplest to analyze. The fastest methylating lysine is K97 (81.8% ^{13}C incorporation) and can be assigned to NMR peak 7 with 78.3%

Table 2. Normalized Percentages of ^{13}C Incorporation for Each Peptide Containing Either One or Two Dimethylamines

lysine/amine in peptide	0.5:1 sample	1:1 sample	2:1 sample	5:1 sample
N-terminal amine, K1	15.4	14.1	17.9	44.8
K13	12.1	17.7	33.6	71.5
K33	10.5	11.5	27.6	64.5
K96, K97		13.2	23.0	52.1
K97	15.5	19.8	36.0	81.8
K116	5.5	11.0	24.2	56.9
K96 (back-calculated)		4.7	11.2	23.7

incorporation. Peaks 1 and 3 have very similar incorporation values of 71.4 and 70.3%, respectively, and either can be assigned to K13 (71.5%). Likewise, the values for peaks 2 and 5 are comparable (61.1 and 60.0%) and can be assigned to either K33 (64.5%) or K116 (56.9%).

MS data for K96 occur only in combination with K97. Since trypsin is not efficient at cleaving at dimethyllysine, these lysines remain together in the dominant tryptic peptide, and their MS data are an average of the two sites. As it turns out, a small percentage of unmethylated and monomethylated sites do exist in the protein, and a small amount of cleaved peptide is actually present giving data separately for K97. The value for K96 can, therefore, be back-calculated using MS data for K97 and the K96−K97 pair. K96 turns out to be the slowest methylating site, with 23.7% ^{13}C incorporation, and can be assigned to peak 6 (30.2%). By process of elimination, the N-terminal amine and K1 must be assigned to peak 4 and one of the peaks 1 or 3. The N-terminal dimethylamine peak can be distinguished from the other peaks by the presence of a strong NOE to the Hα of the N-terminal amino acid (data not shown). On the basis of the appearance of such an NOE, peak 4 can be assigned to the N-terminal dimethylamine and peaks 1 or 3 to K1. Hence, we have been able to make definitive assignments for three of the seven peaks and reduce ambiguities to a pairwise assignment for the remaining four. It is possible that running the methylation reaction at a different pH could remove this final ambiguity.

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