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A liquid chromatography tandem mass spectroscopy approach for quantification of protein methylation stoichiometry

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ABSTRACT

Post-translational modifications are biologically important and wide-spread modulators of protein function. Although methods for detecting the presence of specific modifications are becoming established, approaches for quantifying their mol modification/mol protein stoichiometry are less well developed. Here we introduce a ratiometric, label-free, targeted liquid chromatography tandem mass spectroscopy-based method for estimating Lys and Arg methylation stoichiometry on post-translationally modified proteins. Methylated Lys and Arg were detected with limits of quantification at low fmol and with linearity extending from 20 to 5000 fmol. This level of sensitivity allowed estimation of methylation stoichiometry from microgram quantities of various proteins, including those derived from either recombinant or tissue sources. The method also disaggregated total methylation stoichiometry into its elementary mono-, di-, and tri-methylated residue components. In addition to being compatible with kinetic experiments of protein methylation, the approach will be especially useful for characterizing methylation states of proteins isolated from cells and tissues.

Introduction

Protein methylation is a post-translational modification implicated in the control of gene expression and other functions [1]. Although extensively characterized in the context of nuclear core histone proteins, the majority of methylation occurs on non-histone proteins [2]. For example, the microtubule-associated protein tau is methylated on ≥17 Lys and Arg residues when isolated from mammalian brain [3,4]. "Bottom-up" proteomic approaches indicate that the tau methylation signature varies with disease state, and is positioned to modulate tau aggregation propensity and turnover [3,5]. However, the full functional implications of these observations remain unknown in part because biological effects are mediated by modification stoichiometry, and bottom-up approaches alone capture site distribution but not occupancy. Characterization of complex methylation substrates such as tau would benefit from a "top-down" method [6] capable of capturing overall stoichiometry in terms of quantity (mol methyl equivalents per mol of protein) and quality (chemical form) of methylation under various biological conditions.

The classic top-down approach for characterizing bulk methylation stoichiometry from an ensemble of intact proteins is amino acid analysis, which leverages the unique stability of Lys [7], Arg [7,8], and their methylated derivatives 1meK, 2meK, 3meK, 1meR and 2meR (composed of both ADMA, N^G,N^G-Dimethyl-L-arginine; and SDMA, N^G,$N^{G\prime}$-Dimethyl-L-arginine) upon acid hydrolysis [9,10]. In contrast, other Lys and Arg derivatives are unstable and reduce to the parent amino acids under these conditions. For example, the most prevalent stable mammalian Lys modifications yield amide linkages through acylation (*e.g.*, acetylation, etc; [11]) and isopeptide linkages through conjugation with ubiquitin-like proteins [12], both of which readily hydrolyze in parallel with peptide bonds. Similarly, ADP-ribosylation of Arg, which is mediated by *N,O*-acetal linkages, also is acid labile. As a result, it has been possible to estimate Lys and Arg methylation stoichiometries for specific proteins including calmodulin [13] and myelin basic protein (MBP) [14–17]. Nonetheless, limitations of amino acid analysis have hampered its general application. First, simultaneous separation of Lys, Arg, and all of their methylated derivatives by liquid chromatography is problematic [10], and so methylation stoichiometry

Abbreviations used: 1meK, 2meK and 3meK, N^ϵ;-(methyl)-, N^ϵ;-(dimethyl)-, and N^ϵ;-(trimethyl)-L-lysine, respectively; 1meR, N^G-Methyl-L-arginine; ADMA, N^G,N^G-Dimethyl-L-arginine; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MBP, myelin basic protein; MRM, multiple reaction monitoring; PTM, post-translational modification; PVDF, polyvinylidene fluoride; SDMA, N^G,$N^{G\prime}$-Dimethyl-L-arginine

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has been reported primarily for proteins of low methylation complexity such as calmodulin and MBP (calmodulin contains 3meK and no other methylated residue [13], whereas MBP methylation is limited to 1meR and 2meR [14–17]). Second, amino acid analysis requires derivitization for quantification, which yields poor sensitivity with classic colorimetric agents such as ninhydrin [18]. Replacement with fluorometric detection greatly improves sensitivity [9,10], but even mid-pmol quantification is not adequate for characterizing proteins isolated in low abundance from tissues. Moreover, common derivitization agents such as *o*-pthalaldehyde (OPA) yield Lys adducts that dimerize and quench [19], thereby lowering detection sensitivity for this amino acid still further. Alternative top-down approaches are confined to samples where the complexity of methylation is modest [20].

Here we introduce a targeted mass spectrometric approach for quantifying protein Lys and Arg methylation stoichiometry. The method leverages the established stability of methylated amino acids to acid hydrolysis, but then uses LC-MS/MS to quantify analytes with high accuracy and sensitivity. Because targeted precursor/product ion pairs are detected by a triple-quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode, the approach yields simultaneous and sensitive detection of even complex analyte mixtures. The final assay successfully quantified the methylation stoichiometry of multiply modified, tissue-derived proteins.

Experimental

Materials

Recombinant human 2N4R tau was prepared as described previously [3]. All other reagents were obtained from commercial vendors, including Sigma-Aldrich (St Louis, MO) for calmodulin from bovine testes (P1431), MBP from bovine brain (M1891), unfractionated whole histone from calf thymus (H9250), PVDF membrane (IPVH00005), 1meR (M7033), ADMA (D4268), SDMA (D0390), and all unmodified L-amino acids, and Chem-Impex Intl (Wood Dale, IL) for amino acids 1meK, 2meK and 3meK.

Reductive tau methylation

Recombinant human 2N4R tau was reductively methylated with $NaBH_3CN$ and formaldehyde as described previously [3,21]. Reactions were quenched by addition of glycine after 0, 7, 15, 30 and 60 min incubation. Non-methylated controls were processed identically, except that formaldehyde was omitted from the reaction.

SDS PAGE and blotting

Tau proteins were electrophoresed through 8% acrylamide gels, then transferred (100 V for 1 h at 4 °C) to PVDF membranes in Transfer Buffer A (25 mM Tris, 0.2 mM glycine, 10% methanol). PVDF membranes were then stained (50% Methanol, 7% Acetic Acid, 0.1% Coomassie Brilliant Blue) for 30 min at room temperature with agitation, and then destained (50% methanol, 7% acetic acid) for ~10 min at room temperature with agitation until the staining pattern was visible. After membranes dried at room temperature, Coomassie-blue stained bands were excised using a razor blade (2 × 6 mm slices) and stored at −20 °C until used.

MBP, unfractionated histones, and calmodulin were subjected to SDS-PAGE as above, except that electrophoresis was performed through 15% acrylamide gels. MBP and unfractionated histones were then transferred (100 V for 1 h at 4 °C) to PVDF membranes in Transfer Buffer B (25 mM Tris-HCl, 0.2 mM glycine, 20% methanol), whereas calmodulin was transferred (100 V for 1 h at 4 °C) to PVDF in Transfer Buffer C (25 mM Tris, 0.2 mM glycine, 2 mM $CaCl_2$, 20% methanol) as reported previously [22]. Staining and band excision were performed in the same way as described above for tau proteins.

Acid hydrolysis

Excised PVDF membranes were placed in glass tubes containing 1 mL 6 M HCl and purged with nitrogen as described previously [23]. Samples were then sealed and incubated for 24 h at 125 °C. Following hydrolysis, samples were dried under nitrogen at 60 °C and then stored at −20 °C until LC-MS/MS analysis.

LC-MS/MS quantification

Dried hydrolysates were resuspended in 250 µL of 10 mM HCl, vortexed, filtered (0.2 µm Pall nanosep Mf operated at 14,000*g* for 10 min at 4 °C), and finally prepared for LC by 10-fold dilution with water in glass vials. LC separations were performed on a Hypercarb column (100 × 2.1 mm, 5 µm pore; Thermo Fisher Scientific) operated at 0.2 mL/min using a mobile phase prepared from mixtures of Solvent A (acetonitrile containing 0.1% formic acid) and Solvent B (water containing 0.1% formic acid). The gradient expressed in terms of %-Solvent A was: 0–1 min, 0%; 1–1.1 min, 20%; 1.1–2.7 min, 45%; 2.7–5 min, 60%; 5–5.1 min 90%; 5.1–7 min, 90%; 7–7.1 min, 0%; 7.1–10 min, 0% [24].

Mass spectra were acquired on a triple-quadrupole QTRAP 5500 (AB Sciex) using turbo spray ionization at 2.5 kV in positive ion mode and MRM of parent and characteristic product ions (the transitions monitored are listed in Table 1). The curtain gas (nitrogen) and the collision-activated dissociation were set to 30 psi and medium, respectively. The MS was set to have a dwell time of 35 ms. Analyst 1.6.1 software was used to acquire and process all data.

Table 1
Mass spectrometry parameters and calibration of amino acids.

Amino Acid	Precursor ion	Product ion	Retention time[a]	DP[b]	EP[b]	CE[b]	CXP[b]	Linear range	r^2	LoD[c]	LoQ[c]
	(mass)	(mass)	(min ± SD)	(volts)	(volts)	(volts)	(volts)	(fmol)		(fmol)	(fmol)
Lysine and derivatives											
Lys	147	84	1.16 ± 0.01	40	10	24	12	20–5000	0.995	1.30	4.35
1meK	161	84	1.27 ± 0.02	70	10	23	10	20–5000	0.998	0.56	1.85
2meK	175	84	1.39 ± 0.07	70	10	25	10	20–5000	0.999	0.51	1.69
3meK	189	84	1.46 ± 0.08	70	10	29	10	20–5000	0.999	0.42	1.41
Arginine and derivatives											
Arg	175	70	3.27 ± 0.01	60	10	33	10	20–5000	0.999	0.20	0.65
1meR	189	70	3.27 ± 0.01	50	10	31	8	20–5000	0.999	0.56	1.85
2meR	203	70	3.26 ± 0.01	46	10	33	10	20–5000	0.999	0.24	0.80

[a] Mean ± SD of eight biological replicates collected over a six-month period.
[b] DP; declustering potential; EP, entrance potential; CE, collision energy; CSP, collision cell exit potential as optimized by Analyst 1.6.1 software.
[c] Limits of detection (LoD) and quantification (LoQ) were extrapolated from linear regression analysis as signal-to-background ratios of 3:1 and 10:1, respectively [30].