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MS^3 fragmentation patterns of monomethylarginine species and the quantification of all methylarginine species in yeast using MRM^3 ☆

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ABSTRACT

Protein arginine methylation is one of the epigenetic modifications to proteins that is studied in yeast and is known to be involved in a number of human diseases. All eukaryotes produce Nη-monomethylarginine (ηMMA), asymmetric Nη1, Nη1-dimethylarginine (aDMA), and most produce symmetric Nη1, Nη2-dimethylarginine (sDMA) on proteins, but only yeast produce Nδ-monomethylarginine (δMMA). It has proven difficult to differentiate among all of these methylarginines using mass spectrometry. Accordingly, we demonstrated that the two forms of MMA have indistinguishable primary product ion spectra. However, the secondary product ion spectra of δMMA and ηMMA exhibited distinct patterns of ions. Using incorporation of deuterated methyl-groups in yeast, we determined which secondary product ions were methylated and their structures. Utilizing distinct secondary product ions, a triple quadrupole *multiple reaction monitoring cubed* (MRM^3) assay was developed to measure δMMA, ηMMA, sDMA and aDMA derived from hydrolyzed protein. As a proof-of-concept, δMMA and ηMMA were measured using the MRM^3 method in wild type and mutant strains of *Saccharomyces cerevisiae* and compared to the total MMA measured using an existing assay. The MRM^3 assay represents the only method to directly quantify δMMA and the only method to simultaneously quantify all yeast methylarginines.

Abbreviations: aDMA, asymmetric Nη1,Nη1-dimethylarginine; AdoHcy, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine; [*methyl*-CD$_3$] AdoMet, S-adenosyl-L-[*methyl*-CD$_3$]-methionine; CID, collision-induced dissociation; CPM, counts per min; HPLC, high performance liquid chromatography; LC, liquid chromatography; LIT, linear ion trap; LC-MS/MS, liquid chromatography triple quadrupole mass spectrometry; δMMA, Nε-monomethylarginine, also Nδ-monomethylarginine; ηMMA, Nη-monomethylarginine; MRM, multiple reaction monitoring; MRM^3, multiple reaction monitoring cubed; MS^2, primary product ion spectrum; MS^3, secondary product ion spectrum; MS, mass spectrometry; PRMT, protein arginine *N*-methyltransferase; sDMA, symmetric Nη1,Nη2-dimethylarginine; TFA, trifluoroacetic acid; UHPLC, ultra high performance liquid chromatography; YEPD, yeast extract peptone dextrose.

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1. Introduction

Post-translational modifications to proteins that result in changes to chromatin structure and recruitment of transcription factors assist in regulating gene expression and are part of the growing field of epigenetics. Some of these post-translational modifications were initially studied in yeast and many of the enzymes catalyzing epigenetic modifications to proteins in mammals have yeast homologs. This has led some researchers to use yeast as a model organism for studying epigenetic post-translational modifications, one of which is protein arginine methylation. In humans, the enzymes that catalyze this modification are called protein arginine *N*-methyltransferases (PRMTs). PRMTs are involved in a number of diseases, most notably cancer. The first evidence supporting a role for PRMTs in cancer was the finding that PRMT1 participates in a mixed lineage leukemia complex [1]. More recently, PRMTs have also been found to regulate the expression of tumor suppressors [2–4].

Within the yeast *Saccharomyces cerevisiae*, the enzymes that catalyze arginine methylation are known as arginine methyltransferases (Rmts[1]). To date three Rmts have been identified that catalyze the transfer of methyl groups from the co-substrate S-adenosyl-L-methionine (AdoMet) to the guanidino nitrogen atoms of arginine residues within proteins, producing a methylated protein and the co-product S-adenosyl-L-homocysteine (AdoHcy). Rmt1p (also known as Hmt1p for hnRNP methyltransferase 1) is the principal Rmt within yeast, catalyzing the formation of Nη-monomethylarginine (ηMMA) and asymmetric Nη1, Nη1-dimethylarginine (aDMA) (Fig. 1) [5,6]. The human homologue of Rmt1p called PRMT1 also produces ηMMA and aDMA, and such enzymes are said to have type I activity [7]. The Rmt histone synthetic lethal 7 (Hsl7p) catalyzes the formation of ηMMA in yeast [8], but has also been shown to form symmetric Nη1, Nη2-dimethylarginine (sDMA) with extended incubation times on calf thymus histone H2A using an *in vitro* methylation assay [9] (Fig. 1). It remains unclear if Hsl7p produces sDMA *in vivo* on yeast proteins. The human homologue of Hsl7p, PRMT5, produces both ηMMA and sDMA *in vivo* and is classified as having type II activity. If it is found that Hsl7p only produces ηMMA *in vivo* on yeast proteins, it might be better classified as possessing type III activity (Fig. 1). Rmt2p catalyzes the formation of Nε-monomethylarginine, which is also referred to as Nδ-monomethylarginine (δMMA). Throughout this manuscript we will refer to this methylarginine as δMMA because the name is in wide use, however, we note that according to the IUPAC naming convention for amino acids, the position of methylation for δMMA is Nε. When referring to the positions of atoms within methylarginines we will use the IUPAC convention as delineated in (Fig. 1) [10]. Enzymes that catalyze the formation of δMMA are said to have type IV activity (Fig. 1). Unique among yeast post-translational modifications, δMMA has not yet been discovered in mammalian proteins and at this time Rmt2p has no mammalian homologue. The nearest human equivalent to Rmt2p by sequence similarity is guanidinoacetate *N*-methyltransferase, which is smaller than Rmt2p because it does not have a protein-binding site [11]. Clarke and coworkers were the first to identify δMMA within yeast proteins and the enzyme responsible for its formation, Rmt2p [11,12]. It is unknown whether δMMA is formed on yeast histones and can therefore be inherited as with other epigenetic histone modifications. Nevertheless, Rmt2p does methylate the ribosomal protein L12 of *Saccharomyces cerevisiae* [13]. Another as yet unclassified yeast arginine methyltransferase belonging to the SPOUT methyltransferase family is encoded by the gene YOR021C. It has been found that this gene product demonstrated AdoMet-dependent protein arginine methylation activity and is capable of producing ηMMA. The ribosomal subunit protein Rps3 is methylated by YOR021C at R146, which may affect ribosome function [14].

An interesting consequence of protein arginine methylation in humans is the effect of the free methylarginines when the proteins containing these post-translational modifications are naturally broken down. Both ηMMA and aDMA are inhibitors of nitric oxide synthases that can lead to vasoconstriction and high blood pressure. As a result of their effects on the circulatory system, it is now thought that ηMMA and aDMA are bioactive components in cardiovascular disease [15]. Although not an inhibitor itself, the metabolite of δMMA, Nε-methyl-L-citrulline is a weak non-specific inhibitor of nitric oxide synthases. In addition, δMMA is hydroxylated by human nitric oxide synthases to Nη-hydroxy-Nε-methyl-L-arginine, which is an inhibitor of arginase [16]. These actions of δMMA may have

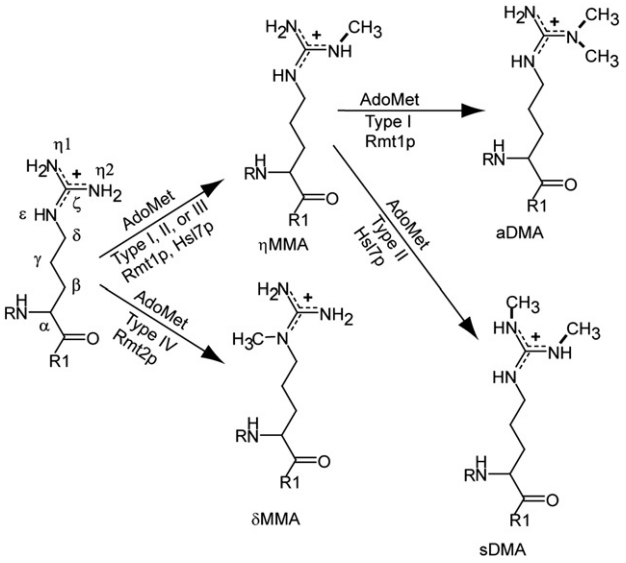

Fig. 1 – Methylated arginines produced in *Saccharomyces cerevisiae*. Shown are the methylated arginines catalyzed by yeast Rmt enzymes. Type I enzymes produce ηMMA and aDMA, Type II produce ηMMA and sDMA, Type III produce only ηMMA, and Type IV produce δMMA. Hsl7p is only known to produce sDMA *in vitro*. The R and R1 groups represent the N- and C-terminal segments of a yeast protein to emphasize that these post-translational modifications are found within proteins. AdoMet is required for each methylation and AdoHcy is produced as a result (not shown).

[1] Yeast genes are represented with capital letters in italics (e.g., *RMT1*), and their corresponding proteins are designated with only the first letter capitalized and ending in the letter "p" to denote that it is a protein (i.e., Rmt1p). Gene deletions are represented with italicised small caps with a superscript minus sign after as in *rmt1*$^{-}$.