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δ-*N*-Methylarginine Is a Novel Posttranslational Modification of Arginine Residues in Yeast Proteins*

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We have found a novel modification of protein arginine residues in the yeast *Saccharomyces cerevisiae*. Intact yeast cells lacking *RMT1*, the gene encoding the protein ω-N^G-arginine methyltransferase, were labeled with the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The protein fraction was acid-hydrolyzed to free amino acids, which were then fractionated on a high resolution sulfonated polystyrene cation exchange column at pH 5.27 and 55 °C. In the absence of the ω-N^G,N^G-[^{3}H]dimethylarginine product of the *RMT1* methyltransferase, we were able to detect a previously obscured ^{3}H-methylated species that migrated in the region of methylated arginine derivatives. The [^{3}H]methyl group(s) of this unknown species were not volatilized by treatment with 2 M NaOH at 55 °C for up to 48 h, suggesting that they were not modifications of the terminal ω-guanidino nitrogen atoms. However, this base treatment did result in the formation of a new ^{3}H-methylated derivative that co-chromatographed with δ-*N*-methylornithine on high resolution cation exchange chromatography, on reverse phase high pressure liquid chromatography, and on thin layer chromatography. From these data, we suggest that the identity of the original unknown methylated residue is δ-*N*-monomethylarginine. The presence of this methylated residue in yeast cells defines a novel type of protein modification reaction in eukaryotes.

The activity of many proteins is modulated by the covalent posttranslational modification of specific amino acid residues. Some of these modification reactions, such as phosphorylation, are reversible whereas others appear to permanently modify residues effectively enlarging the repertoire of amino acids available to proteins. A major group of the latter reactions involves *S*-adenosylmethionine (AdoMet)[1]-dependent methylation of the side chain nitrogen atoms of histidine, lysine, and arginine residues (1–3). Recent interest has focused on the formation of several methylated arginine derivatives in a variety of eukaryotic proteins involved in signal transduction, nuclear RNA processing, the structural integrity of myelin, and other functions (4–11). There are at least three distinct types of protein arginine *N*-methyltransferases that have been classified by their reaction products. The type I enzymes catalyze the formation of ω-N^G-monomethylarginine and asymmetric ω-N^G,N^G-dimethylarginine residues, the type II enzymes catalyze the formation of ω-N^G-monomethylarginine and symmetric ω-N^G,N'^G-dimethylarginine residues, whereas the type III enzyme forms only the ω-N^G-monomethylarginine derivative (for a review, see Ref. 11). The precise functional role of these methylated arginine residues has not been established.

We have been interested in studying the modification of yeast proteins by methylation. The complete genome of *Saccharomyces cerevisiae* has been sequenced (12), and the presence of methyltransferase sequence motifs in many of these enzymes aids in the identification of new modification enzymes (13). Yeast cells, in contrast to almost all other cell types, actively import the biological methyl donor *S*-adenosylmethionine (14), and this allows radiolabeling of proteins in intact cells under *in vivo* conditions to determine the relevant substrates. Finally, yeast knockout mutants can be readily constructed and can be useful in the pairing of specific methyltransferase genes with their substrates.

We recently identified the gene responsible for the majority of protein arginine methylation in yeast (5). This gene, *RMT1*, encodes a type I protein arginine methyltransferase capable of sequentially methylating arginine residues to ω-N^G-monomethylarginine and ω-N^G,N^G-dimethylarginine in a variety of proteins (5, 11). Mutants lacking this enzyme appear to be completely deficient in the formation of ω-N^G,N^G-dimethylarginine residues, suggesting that this enzyme is wholly responsible for this modification. However, these mutants contain residual ω-N^G-monomethylarginine residues as well as an additional unknown methylated residue that chromatographs between the positions of the dimethyl- and monomethylarginine derivatives (5).

In this paper, we provide evidence that this latter methylated residue represents a novel modification of arginine residues giving rise to δ-*N*-monomethylarginine through the methylation of the internal (or δ) guanidino nitrogen atom and suggest that a new type of protein arginine methyltransferase exists in nature.

EXPERIMENTAL PROCEDURES

Isotopic Labeling—S. cerevisiae strain JDG9100–2 (*MAT***a**, *prc1–407, prb1–1122, pep4–3, leu2, trp1, ura3–52, ycl57w*Δ*::URA3, rmt1::LEU2*) (5) was grown at 30 °C to early log phase (approximately 5×10^7 cells/ml; $A_{600\ nm} = 0.8$) in YPD medium (1% (w/v) yeast extract (Difco Laboratories), 2% (w/v) bacto-peptone (Difco), and 2% (w/v) D-glucose). Cells from a 6-ml culture were harvested at 1000 × *g* for 3 min in a 15-ml polystyrene centrifuge tube at room temperature. Cells were washed 3 times with 1 ml of YPD medium and resuspended in 0.82 ml of YPD medium. The suspension was transferred to a 1.5-ml polypropylene microcentrifuge tube and mixed with 0.18 ml of [^{3}H]AdoMet (Amersham Life Science, 79 Ci/mmol, 1 mCi/ml in dilute HCl (pH 2.0–2.5):ethanol (9:1, v/v) (final concentration of 1.4 μM) and incubated while shaking at 30 °C for 30 min. Cells were then pelleted at 13,600 × *g* at room temperature for 1 min, washed twice with 1 ml of water, and used immediately or stored at −80 °C.

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[1] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; HPLC, high pressure liquid chromatography.

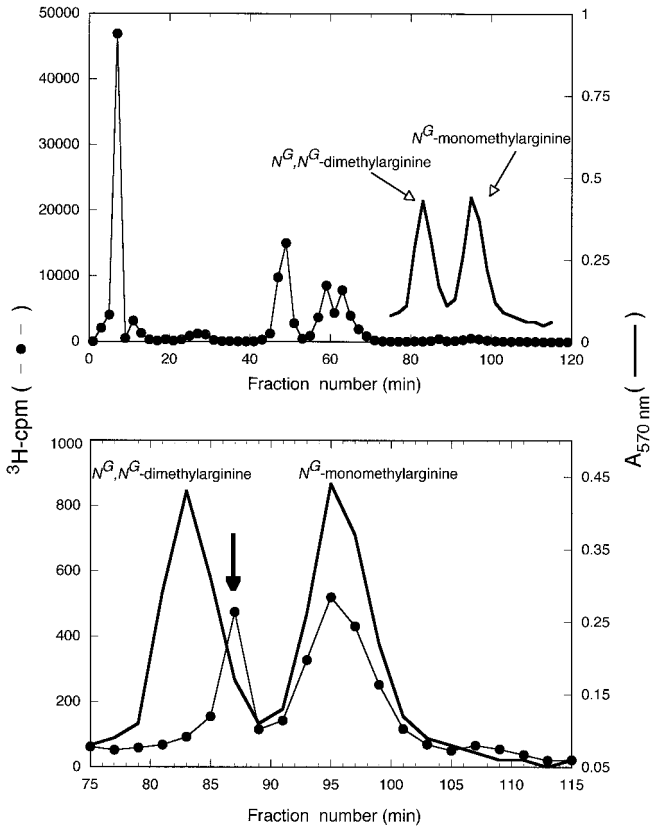

FIG. 1. **Amino acid analysis of a radiolabeled extract from a protein arginine methyltransferase-deficient yeast mutant reveals a distinct methylated product.** *S. cerevisiae* strain *JDG9100–2* was isotopically labeled *in vivo* with [^{3}H]AdoMet as described under "Experimental Procedures." Precipitated proteins from a cell lysate were acid-hydrolyzed (see "Experimental Procedures"), resuspended in water, and mixed with 1 μmol each of the standards ω-N^G,N^G-dimethylarginine (asymmetric) and ω-N^G-monomethylarginine (both obtained from Sigma). An equal volume of Pierce citrate sample dilution buffer (0.2 M in Na^+ containing 2% thiodiglycol and 0.1% phenol at pH 2.2) was then added, and the resulting mixture was loaded onto an amino acid analysis cation exchange column (Beckman AA-15 sulfonated polystyrene, 0.9-cm diameter × 11-cm height) equilibrated with sodium citrate buffer (0.35 M in Na^+, pH 5.27) at 55 °C and eluted at 1 ml/min. One-min fractions were collected, and ^{3}H radioactivity (*filled circles*) was determined by counting a 200-μl aliquot of every other fraction diluted with 2 volumes of water in 5 ml of fluor. An additional 100 μl of these fractions was analyzed for the non-isotopically methylated amino acid standards using the ninhydrin assay (5) (*solid line*). The *upper panel* represents the entire chromatograph; in the *lower panel*, the region corresponding to methylated arginine derivatives is enlarged. The column was regenerated with 0.2 N NaOH.

Preparation of Cell Extracts—Isotopically labeled cells were resuspended in 50 μl of 1% sodium dodecyl sulfate (w/v) and 0.67 mM phenylmethylsulfonyl fluoride. Acid-washed glass beads (0.5 mm diameter, Biospec Products) were then added (0.2 g) to the cell suspension, and the tube was vortexed for 1 min followed by an incubation on ice for 1 min. This cycle was repeated 7 times. Approximately 40 μl of this extract was removed with a micropipette tip and placed into a new tube. The beads were washed with an additional 50 μl of the lysis solution and combined with the cell lysate.

Acid Hydrolysis of ^{3}H-Labeled Cell Lysate—Aliquots (30 μl) of the cell lysate were mixed with an equal volume of 25% (w/v) trichloroacetic acid in a 6 × 50-mm glass vial and incubated at room temperature for 20 min. The precipitated material was pelleted at 4000 × *g* for 40 min at 25 °C. The pellets were washed once with acetone (100 μl) at −20 °C, dried, and then acid-hydrolyzed in the presence of 200 μl of 6 N HCl at 110 °C for 20 h in a Waters Pico-Tag vapor-phase apparatus. The hydrolyzed samples were resuspended in 200 μl of water for analysis by cation exchange chromatography.

Synthesis and Characterization of δ-N-Methylornithine and Derivatives—δ-*N*-Methylornithine was synthesized after the general reductive methylation strategy described by Benoiton (15). α-*N*-Acetyl-L-ornithine (29 μmol, Sigma) was dissolved in 280 μl of 1 M NaOH in a 12 × 75-mm borosilicate test tube. A molar equivalent of formaldehyde (2.5 μl of 37% (w/w), Fisher) was then added. The reaction proceeded at room temperature, and the solution was mixed periodically over a 20-min period. *N*-Acetylation of the α-nitrogen atom of the substrate ensured that only the δ-nitrogen atom of ornithine would be modified. Four molar equivalents (116 μmol; 116 μl) of freshly prepared $NaBH_4$ dissolved in 1 M NaOH were added to the mixture, vortexed, and incubated at 15 °C for 1 h with periodic mixing. Approximately 150 μl of 6 N HCl was added dropwise to the mixture until the pH of the solution was less than 2. Aliquots (100 μl) were placed into 6 × 50-mm glass vials and dried under vacuum at room temperature using a Speed Vac (Savant). The sample was then acid-hydrolyzed to remove the α-*N*-acetyl protection group, as described above. The material in each tube was dissolved in 50 μl of water and then combined and stored at −20 °C.

TABLE I
Base-dependent volatility of methyl arginine derivatives

Pooled fractions from the cation exchange column representing ω-N^G,N^G-[*methyl*-^{3}H]dimethylarginine, ω-N^G-[*methyl*-^{3}H]monomethylarginine, and the novel ^{3}H-methylated peak were analyzed for volatile radiolabeled products after base treatment. Aliquots (200 μl) were mixed with NaOH (final concentration of 1 or 2 M) in microcentrifuge tubes and placed (lids open) into scintillation vials containing 5 ml of fluor (SafetySolve, Research Products Inc.). The vials were capped tightly and placed into a 55 °C oven for 24 or 48 h to allow diffusion of volatile products from the vial to the fluor. The vials were then removed and counted for radioactivity. All values are given as averages of duplicate determinations.

^{3}H-Methylated amino acid	Volatility		
	1 M NaOH (24 h)	1 M NaOH (48 h)	2 M NaOH (24 h)
		%	
ω-N^G,N^G-Dimethylarginine	44.9	53.3	99.3
ω-N^G-Monomethylarginine	21.6	28.0	47.7
Novel derivative	7.4	8.3	10.3

The reaction products were analyzed by thin layer chromatography at room temperature on a 20-cm sheet coated with a 0.2-mm layer of silica 60 (EM Separations, Gibbstown, NJ, no. 5748) with a mobile phase consisting of CH_3OH:~14.8 N NH_4OH (3:1, v/v). After heating the sheet at 105 °C in a vacuum oven for 5 min to evaporate the solvents, it was sprayed with ninhydrin (10 mg/ml in acetone) and incubated at 55 °C. Three ninhydrin-positive spots were detected at R_F values of 0.37, 0.42, and 0.63. The areas corresponding to these spots on an adjacent lane were scraped into a microcentrifuge tube and submitted to the UCLA Center for Molecular and Medical Sciences Mass Spectroscopy for electrospray analysis. The samples in each tube were dissolved in 400 μl of H_2O:CH_3CN (50:50, v/v) and centrifuged momentarily to pellet silica debris. An aliquot (20 μl) was mixed with 10 μl of H_2O:CH_3CN:HCOOH (50:50:0.1, v/v) and scanned in positive ion mode from 120 to 220 *m/z*. We found that the compound migrating at R_F = 0.42 had a mass of 133 corresponding to ornithine, the R_F = 0.37 compound with a mass of 147 corresponds to δ-*N*-monomethylornithine, and the R_F = 0.63 compound with a mass of 161 corresponds to δ-*N,N*-dimethylornithine.

The reaction products were purified on a preparative basis using the cation exchange column described above. Two ninhydrin-positive peaks were detected at 43–47 min and 50–55 min that were identified by thin layer chromatography as ornithine and as a mixture of δ-*N*-monomethylornithine and δ-*N,N*-dimethylornithine, respectively. To separate the mono- from the dimethylornithine species, the 50–55-min peak was pooled, and 1 ml was injected onto a reverse phase HPLC column (Econosphere C18 5 μm column from Alltech, 4.6 mm by 250 mm) equilibrated in 0.1% trifluoroacetic acid/H_2O at room temperature. One-min fractions were dried under vacuum, and pellets were resuspended in 10 μl of water and analyzed by thin layer chromatography as described above. δ-*N*-Monomethylornithine was found to elute within the first 8 min whereas the bulk of δ-*N,N*-dimethylornithine elutes between 8 and 11 min.

We also performed this synthesis with [^{3}H]$NaBH_4$ to obtain radiolabeled methyl derivatives. The same procedure was used except 4.4 μmol of sodium [*methyl*-^{3}H]borohydride (NEN Life Science Products, 222 mCi/mmol dissolved in 1 M NaOH, pH 9) was used with 4.4 μmol of $NaBH_4$. Thin layer chromatography showed radioactivity in species at R_F = 0.33 and R_F = 0.60 corresponding to δ-*N*-monomethylornithine and δ-*N,N*-dimethylornithine, respectively.

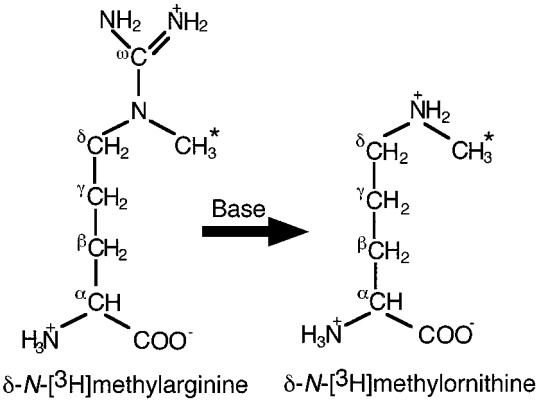

FIG. 2. **δ-*N*-[*methyl*-^{3}H]Methylarginine yields δ-*N*-[*methyl*-^{3}H]-methylornithine upon base treatment.**

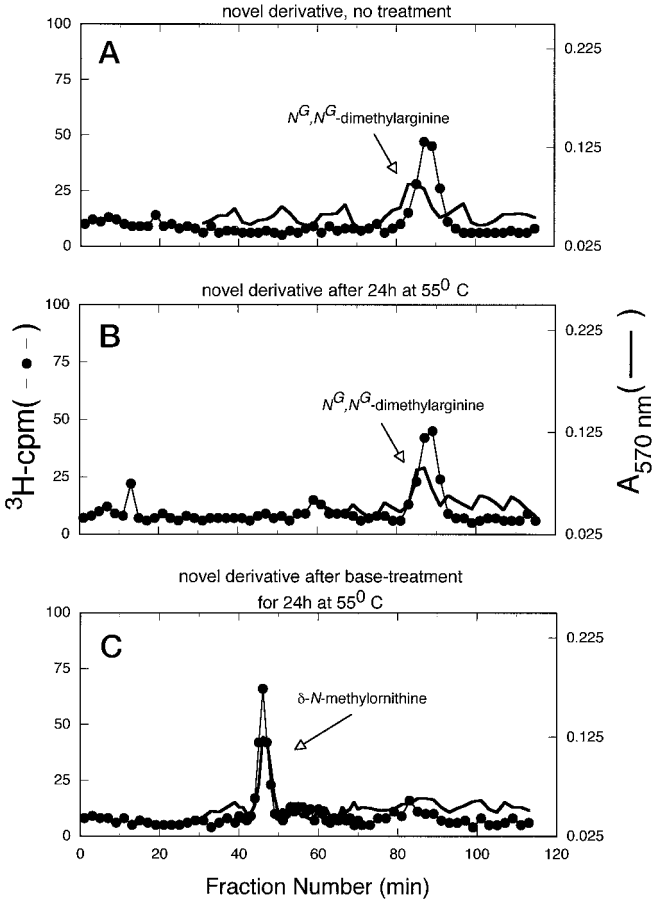

FIG. 3. **Base treatment of the novel methylated amino acid derivative produces a species that co-chromatographs with δ-*N*-methylornithine.** Fractions 86–88 encompassing the unknown peak eluting just after the ω-N^G,N^G-dimethylarginine standard peak on the cation exchange column from Fig. 1 were pooled. *A*, an aliquot of the pool (800 μl) was rechromatographed as shown in Fig. 1. *B*, an aliquot of the pool (800 μl) was incubated for 24 h at 55 °C and rechromatographed as above. *C*, an aliquot (800 μl) of the pool was mixed with 10 M NaOH to give a final concentration of 2 M NaOH. The mixture was incubated at 55 °C for 24 h and mixed with 200 μl of 12 N HCl, and 40 μl (~150 nmol) of HPLC-purified δ-*N*-methylornithine was added. The reaction mixture was then diluted with 17 ml of water and rechromatographed as above.

RESULTS AND DISCUSSION

When protein arginine methylation was characterized in a yeast *rmt1* mutant deficient in ω-N^G,N^G-dimethylarginine formation, a peak of residual [^{3}H]methyl radioactivity was found

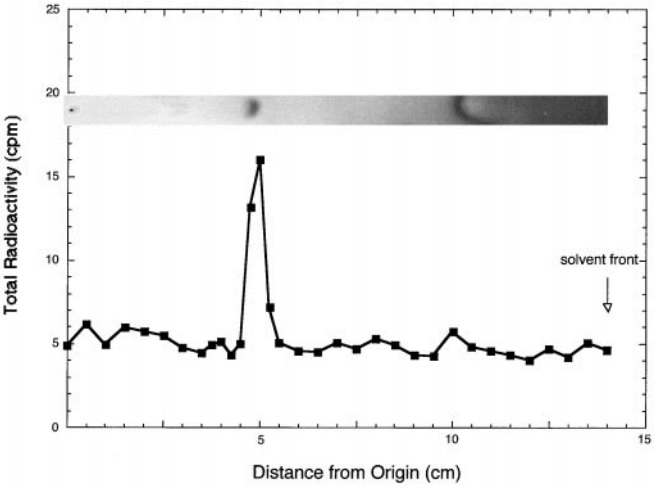

FIG. 4. **Co-migration of the ^{3}H-methylated, base-treated unknown product with δ-*N*-monomethylornithine using thin layer chromatography.** The position of the δ-*N*-monomethylornithine standard at 5 cm is shown in the ninhydrin-stained *lane* at the *top* (the apparent color at 10 cm is a staining artifact). Radioactivity in sections of this *lane* was determined by scraping the silica into scintillation vials, resuspending in 500 μl of water, and counting in 5 ml of fluor for 20 min for a total of 4 cycles.

to elute just after the expected position of ω-N^G,N^G-dimethylarginine (5). We have now investigated whether this material might represent a new type of methylated arginine derivative. Intact yeast cells were incubated with the isotopically labeled biological methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Following disruption of the cells, acid hydrolysates of the protein fraction were applied to a high resolution amino acid analysis cation exchange column along with standards of ω-N^G-monomethylarginine and ω-N^G,N^G-dimethylarginine (Fig. 1). Although the bulk of radioactivity was found in species eluting prior to the methylated arginine derivatives, a well defined peak of ^{3}H radioactivity consistently eluted between the two standards in a position similar to that of the symmetric ω-N^G,N'^G-dimethylarginine derivative.

A diagnostic test for arginine derivatives is their lability to base treatment. Breakdown products of arginine in base include ornithine, citrulline, urea, carbon dioxide, and ammonia (16). Arginine residues methylated at the terminal (ω) nitrogen atoms have been shown to yield methylamine and methylurea (17–20) upon base treatment. Control experiments with ω-N^G,N^G-[^{3}H]dimethylarginine (isolated from experiments similar to that shown in Fig. 1 with wild type yeast; Ref. 5) or with ω-N^G-[^{3}H]monomethylarginine show that a substantial fraction of the radioactivity is converted to a volatile form (such as methylamine or dimethylamine) by treatment with 1–2 M NaOH for 24–48 h (Table I). When the unknown derivative eluting at 86–88 min (Fig. 1) was subjected to base treatment, however, we recovered very little volatile radioactivity (Table I), suggesting that it did not represent any of the known ω-N^G-methylated forms of arginine.

We then considered the possibility that the derivative represented an arginine species methylated on the internal imino nitrogen atom of the guanidino group (*i.e.* δ-*N*-methylarginine) (Fig. 2). Consistent with the results from Table I, base treatment of such a species would produce the non-volatile methylated product δ-*N*-methylornithine (17–19, 21). We therefore synthesized a standard of δ-*N*-methylornithine (see "Experimental Procedures") for comparison with the base-treated product of the unknown ^{3}H-methylated species. A fraction of the unknown ^{3}H-methylated peak from the cation exchange column in Fig. 1 was mixed with NaOH to a final concentration

of 2 M NaOH and incubated at 55 °C for 24 h, as described in Fig. 3. Rather than analyze the volatile radioactivity as in Table I, the base-treated sample was rerun on the cation exchange column and compared with standards to analyze the nonvolatile radioactivity. We found that the radioactivity in the original unknown ^{3}H-methylated peak at 85–91 min disappeared and was quantitatively transferred to a new peak of radioactivity in the 43–47-min region (Fig. 3*C*). This radioactive peak exactly co-eluted with the synthetic δ-*N*-methylornithine added as an internal standard (Fig. 3*C*). As controls, the unknown ^{3}H-methylated peak was stable when rerun on the cation exchange column without heat or base treatment (Fig. 3*A*) or when incubated at 55 °C for 24 h without base (Fig. 3*B*). These studies indicate that the formation of the new ^{3}H-methylated product co-eluting with δ-*N*-methylornithine is dependent upon base treatment.

To confirm the identification of δ-*N*-[*methyl*-^{3}H]ornithine, fractions forming the ^{3}H-methylated peak at 45–49 min in Fig. 3*C* were pooled, and 1 ml was directly injected onto a reverse phase HPLC column as described under "Experimental Procedures." The radioactivity representing the base-treated ^{3}H-methylated unknown peak eluted from the HPLC column in a flow-through peak with the salt and in a distinct peak at 8 min (data not shown). This latter material was dried and subjected to thin layer chromatography as described under "Experimental Procedures." We found that radioactivity from the base-treated ^{3}H-methylated species exactly co-eluted with the δ-*N*-monomethylornithine standard (Fig. 4). These results provide strong evidence that the product of the base treatment is δ-*N*-[^{3}H]monomethylornithine and that [*methyl*-^{3}H]-δ-*N*-monomethylarginine is present in the hydrolysate of the *rmt1* yeast cytosol.

The initial studies of methylated arginine derivatives in proteins focused on the characterization of mono- and dimethylated arginine residues in bovine and rat tissues (17–19, 22, 23). In fact, Paik and Kim (18) specifically ruled out the existence of δ-*N*-methylarginine in hydrolysates of rat liver nuclei when they found that alkaline treatment of guanidino-[*methyl*-^{14}C]arginine derivatives yielded radiolabeled methylurea and methylamine; no radioactive product co-chromatographed with a synthetic δ-*N*-methylornithine standard. Nakajima *et al.* (19) concurred with this result in similar experiments using hydrolysates of proteins from bovine brain and various rat organs. There appear to be no further studies of the possible existence of δ-*N*-methylarginine residues in proteins since these reports were published. In light of our discovery of these residues in yeast proteins, it might now be of interest to ask whether δ-methylarginine residues are indeed present in mammalian cell proteins but at levels where they are obscured by the much more prevalent ω-methylarginine derivatives.

The physiological function served by the methylation of arginine residues at the δ-nitrogen atom is unknown. However, it is clear that such methylation would effectively disrupt the ability of arginine to hydrogen bond at this atom (24, 25) and may thus allow for more specific hydrogen bonding patterns involving the unmethylated ω-nitrogen atoms of the same residue. The determination of the polypeptide(s) in yeast cells that contain δ-*N*-methylarginine residues, as well as the identification of the enzyme(s) that catalyze its synthesis, will be important steps in understanding the role of this modification.

Although there have been no previous reports of the presence of δ-*N*-methylarginine residues in proteins, there is a report in the literature on the existence of the free amino acid in the root tuber of snake gourd (*Trichosanthes cucumeroides)* (26). Additionally, free δ-*N*-methylornithine, the base-generated product of δ-*N*-methylarginine, has been suggested to occur in bovine brain by chromatographic analysis of tissue extracts (21). Furthermore, δ-*N*-methylornithine appears to be a biosynthetic precursor of nicotine in tobacco plants (27). The results presented here open the possibility that these free amino acids may originate from the proteolytic hydrolysis and degradation of proteins containing δ-*N*-methylarginine. In light of the inhibitory properties of ω-*N*-methylarginine derivatives on nitric oxide synthase (28), it would be interesting to ask if δ-*N*-methylarginine has similar effects that may be of physiological relevance in mammals.

Finally, we should point out that there is precedence for the enzymatic methylation of the imino δ-nitrogen atom in a small molecule arginine derivative rather than in protein as seen here. A well characterized methyltransferase (EC 2.1.1.2) converts guanidinoacetate to creatine, its δ-*N*-methyl derivative, in the biosynthetic pathway leading to creatine phosphate in vertebrate and echinoderm cells (29).

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