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N^G-Methylarginines: Biosynthesis, biochemical function and metabolism

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Summary. N^G-Methylarginines (N^G-monomethylarginine, N^G, N^G-dimethylarginine and N^G, N'^G-dimethylarginine) occur widely in nature in either protein-bound or in free states. They are posttranslationally synthesized by a group of enzymes called protein methylase I with S-adenosyl-L-methionine as the methyl donor. The enzymes are highly specific not only towards arginine residues but also towards the protein species. Since transmethylation reaction is energy-dependent in the form of S-adenosyl-L-methionine and is catalyzed a group of highly specific enzymes, it is quite logical to assume that the enzymatic methylation of protein-bound arginine residues play an important role in the regulation of the function and/or metabolism of the protein. When determined with histones as *in vitro* substrates, protein methylase I activity parallels closely the degree of cell proliferation, and the myelin basic protein (MBP)-specific protein methylase I activity decreases drastically in dysmyelinating mutant mouse brain during myelinating period, suggesting an important role played in the formation and/or maintenance of myelin. When the methylated proteins are degraded by intracellular proteolytic enzymes, free N^G-methylarginines are generated. Some of these free N^G-methylarginines, particularly N^G-monomethylarginine, are extensively metabolized by decarboxylation, hydrolysis, transfer of methylamidine and deimination reaction. Recent experiment demonstrates that some of the N^G-methylarginines may be involved in the neutralization of activity of nitric oxide (NO) which has attracted a great deal of attention as vascular smooth muscle relaxation factor.

Keywords: Amino acids – N^G-Methylarginines – Protein-arginine – Metabolism – Biosynthesis – Protein methylase I – NO Antagonist

Opening remarks

Before going into the major topics, a brief account of the genesis of N^G-methylarginines will be presented. While investigating the origin of ε-N-

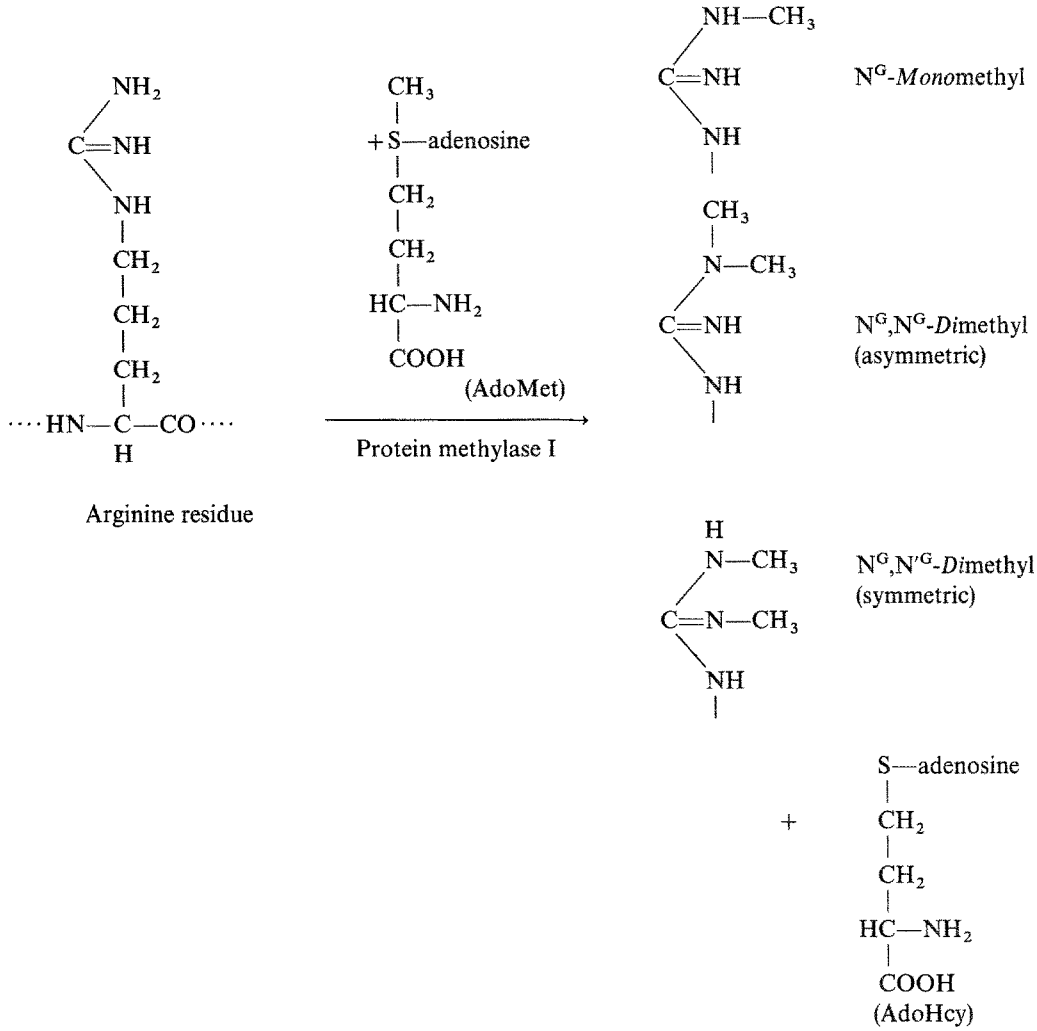
methyllysine in histone molecule, it was realized by paper chromatographic technique that the ε-N-methyllysine observed by many previous investigators was, in fact, not pure but a mixture of ε-N-monomethyllysine and ε-N-dimethyllysine. In 1967, using a modified elution buffer in an automatic amino acid analyzer, the presence of ε-N-dimethyllysine was definitely uncovered in histone hydrolyzate in addition to the already known ε-N-monomethyllysine. A year later the presence of ε-N-trimethyllysine in histone was established, thus completing the discovery of all the possible methyl substituted lysine derivatives. For this reason the identification and purification of the enzyme responsible for the methylation of the ε-NH_2 group of lysine residues was of great importance. Such an enzyme from the cytosol of calf thymus which methylated the endogenous protein and utilized S-adenosyl-L-methionine as the methyl donor was purified. However, this enzyme, which was the first to be discovered and called "protein methylase I", did not turn out to be the lysine methylase as originally sought after. Instead, much "dismay" to us, the amino acid methylated on the protein was arginine and not lysine (Paik and Kim, 1968).

Introduction

In 1968, we first described the presence in calf thymus of an enzyme which transferred methyl groups from S-adenosyl-L-methionine (AdoMet) to the guanidino-N (N^G-) groups of arginine residues in preformed polypeptide chain (here, calf thymus histone), but not to free arginine (Paik and Kim, 1968). Subsequent studies abundantly demonstrated that the enzymatic methylation of protein-bound arginine residues constituted one of the ubiquitously occurring posttranslational modification reactions of protein (Paik and Kim, 1980; 1990). The chemical structure of the products formed was identified to be N^G-monomethyl-L-arginine, N^G, N^G-dimethyl-L-arginine (asym.) and N^G, N'^G-dimethyl-L-arginine (sym.) (Scheme 1) (Paik and Kim, 1968; Kakimoto and Akazawa, 1970). As shown in Tables 1 and 2, these methylated arginine derivatives have been found to occur widely in nature, in many highly specialized proteins as well as in free form.

The enzyme responsible was originally thought to be a single enzyme which was specific towards arginine residues regardless of the protein species. Thus, it was designated as protein methylase I [S-Adenosylmethionine: protein-arginine N-methyltransferase; EC 2.1.1.23]. However, recent observations clearly demonstrate that there is a class of enzymes which are not only specific towards the arginine side chains, but also a second level of specificity towards protein species.

The proteins that are methylated posttranslationally will subsequently be degraded and free methylated arginine derivatives will be released *in vivo*. Since methylated arginines cannot be reincorporated into protein, some of these free N^G-methylarginines will be further metabolized. On the other hand, in higher organisms some of them will find their way into the kidney for eventual disposal by way of the circulatory system. A very important recent observation is that N^G-monomethyl-L-arginine acts as an antimetabolite for the biosynthesis of nitric oxide, an endothelium-derived relaxation factor (Palmer et al., 1987). In



Scheme 1. Reaction products of protein methylase I

this article, we have attempted to review the enzymology involved in the biosynthesis and the metabolism of N^G-methylarginines.

Natural occurrence

N^G-Methylarginines are widely distributed among eukaryotic organisms (Table 1), and it is noteworthy to find that fungi such as *Aspergillus niger* and *Penicillum notatum* contain relatively large amounts of these amino acids. It is also noted that N^G, N^G-dimethylarginine (asymmetric DMA) is by far the most abundant in almost all the eukaryotes, except *Actinomycetes*. However, uncertainty still exists with regards to the presence of methylated arginines in prokaryotic organisms and there is no supporting evidence to show the presence of protein methylase I in prokaryotes. The protein methylase I has been purified from *Euglena gracilis* (protozoon), wheat germ, calf thymus and brain (Paik and Kim, 1985), and human placenta (Paik et al., 1990).

Table 1. The content of various methylated arginine derivatives in the proteins of microorganism, plant, insect, bird and mammal (Matsuoka, 1972)

	N^G-MMA[a]	N^G, N^G-DMA	N^G, N'^G-DMA
Eubacterium:			
Escherichia coli	−[b]	−	−
Actinomycetes:			
Streptomyces griseus	0.49	0.16	+
Streptomyces lavend.	0.92	+	+
Yeast:			
Sacchar. cerevisiae	0.09	0.58	0.05
Brettanomyces	+	0.86	+
Fungus:			
Aspergillus niger	0.66	2.19	+
Penicillum notatum	1.25	5.24	+
Plant:			
Pea (1 day sporulation)	0.08	0.39	0.06
Carrot (root)	0.19	1.27	0.06
Insect:			
Fly (adult)	0.05	0.55	+
Amphibia:			
Tadpole	0.07	1.50	0.10
Adult	0.07	1.66	0.10
Bird:			
Pigeon liver	0.39	1.31	0.16
Mammal:			
Guinea pig brain	0.69	1.41	0.30
Rabbit heart	+	0.44	0.08
Rat brain	0.93	1.64	0.45
Rat intestine	0.42	2.66	0.43
Erythrocyte protein:			
Chicken	0.20	0.76	0.16
Rabbit	−	0.03	−

[a] MMA and DMA represents monomethylarginine and dimethylarginine, respectively.
[b] Minus sign indicates that the amount is not detectable (<0.01 μmoles) and plus sign indicates that the amount is small (0.01–0.04 μmoles).
The values represent μmoles per gram of protein.

Table 2 lists proteins which have been shown to contain various methylated arginine derivatives. Here again, in agreement with the data shown in Table 1, N^G, N^G-dimethylarginine (asymmetric) has the longest list of proteins which contain this amino acid. Amino acid sequence analysis of *Euglena gracilis* histone H1 or cytochrome *c* did not reveal the presence of N^G-monomethylarginine, however, these proteins are highly specific as well as effective as *in vitro* methyl-acceptor for respective enzymes. The presence of N^G, N^G-dimethylarginine in heat shock protein hsp 70 of rice cell culture has been demonstrated during heat-shock treatment.

Concerning the number of N^G-methylarginine-containing proteins occurring in nature, Table 2 might appear to be highly conservative. In order to investigate in depth the scope of endogenous methyl-acceptor proteins, Najbauer

Table 2. Natural occurrence of N^G-methylarginines and some related compounds

Protein	Source	Reference
	N^G-Monomethylarginine	
Histone*	Thymus (calf)	Paik & Kim (1967)
Nuclear acidic protein	Liver nuclei (rat)	Friedman et al. (1969)
Myelin basic protein (MBP)	Myelin (human)	Baldwin & Carnegie (1971)
Heat shock protein	Chicken fibroblast	Wang et al. (1982)
Histone H4*	Wheat germ	Gupta et al. (1982)
Cytochrome *c*	*Euglena gracilis*	Farooqui et al. (1985)
Free amino acid	Seed of *Vicia faba* L. (bean)	Kasai et al. (1976)
	Placenta (human)	Tomita & Nakamura (1977)
	N^G, N^G-Dimethylarginine (asymmetric)	
Histone*	Thymus (calf)	Paik & Kim (1967)
Myelin basic protein	Myelin (human)	Baldwin & Carnegie (1971)
Myosin	Leg muscle (rat and chicken)	Reporter & Corbin (1971)
hnRNP protein (protein A1)	Liver nuclei (rat and chicken)	Boffa et al. (1977)
	Nuclei (*Physarum polycephalum*)	Christensen et al. (1977)
HMG-2	Nuclei (rat)	Boffa et al. (1979)
C23 acidic phosphoprotein	Nucleolus (Novikoff hepatoma)	Lischwe et al. (1982)
Ribosomal proteins	HeLa cells	Chang et al. (1967)
Tooth matrix protein	Human	Kalasz et al. (1978)
Scleroderma antigen	Nucleolus (Novikoff hepatoma)	Lischwe et al. (1985)
Heat shock protein (hsp-70)**	Rice cell (*Oryza sativa*)	Fourre & Lhoest (1989)
Histone H2B**	*Drosophila*	Desrosiers & Tanguay (1988)
Free amino acid	Urine (human)	Kakimoto & Akazawa (1970)
	Serum (human)	Kakimoto & Akazawa (1970)
	Placenta (human)	Tomita & Nakamura (1977)
	Seed of *Vicia faba L.*	Kasai et al. (1976)
	N^G, N'^G-Dimethylarginine (symmetric)	
Histone*	Thymus (calf)	Paik & Kim (1967)
Myelin basic protein	Myelin (human)	Baldwin & Carnegie (1971)
Tooth matrix protein	Human	Kalasz et al. (1978)
Histone H2B**	*Drosophila*	Desrosiers & Tanguay (1988)
Free amino acid	Urine (human)	Kakimoto & Akazawa (1970)
	Serum (human)	Kakimoto & Akazawa (1970)
	Seed of *Vicia faba L.*	Kasai et al. (1976)
	δ-N-Monomethylarginine	
	Root tuber (*Trichosanthes cucumeroides*)	Inukai et al. (1968)
	N^G-Hydroxymethylarginine	
Free amino acid	Serum (human)	Csiba et al. (1986)
	Urine (human)	Csiba et al. (1986)
	δ-N-Methylornithine	
Free amino acid	Brain (bovine)	Matsuoka et al. (1969)

* Demonstrated only during *in vitro* enzymatic methylation.
** Demonstrated only during heat shock treatment.

and Aswad (1990) cultured rat pheochromatoma cells first with adenosine dialdehyde (a methylation inhibitor) to hypomethylate the endogenous proteins, and the extract of the resulting cells was subsequently radiolabeled with S-adenosyl-L-[*methyl*-^{3}H]methionine. On analysis by two-dimensional gel electrophoresis followed by fluorography, over 50 methyl-acceptor proteins were detected. N^G-Monomethylarginine and N^G, N^G-dimethylarginine accounted for about 90% of the total [*methyl*-^{3}H]-labeled amino acids.

Biosynthesis

Methylation of arginine at polypeptide level

N^G-Methylarginines are posttranslationally synthesized at the polypeptide level. Although systematic studies as carried out during the investigation of the origin of ε-N-methyllysines (Kim and Paik, 1965) were not documented in respect to the origin of N^G-methylarginines, highly purified protein methylase I [EC 2.1.1.23] uses S-adenosyl-L-methionine as the sole methyl donor and is insensitive to the action of puromycin. Furthermore, it should be noted that no codon is available for the methylated amino acids because all of the possible 64 codons are already assigned for the amino acids commonly found in protein (Rawn, 1989; Paik and Kim, 1980a).

Multiplicity of the protein methylase I

When first identified, it was assumed that a single enzyme protein methylase I was responsible for methylating *in vivo* all the proteins listed in Table 2. However, recent evidence clearly demonstrates that there are subtypes of protein methylase I which are specific towards protein species in addition to arginine residues. So far, two subtypes of the enzyme have been identified and characterized; namely, myelin basic protein (MBP)-specific and histone-specific (Ghosh et al., 1988) (Table 3). Interestingly, however, very recent studies from this laboratory indicate that the latter enzyme also methylates the recombinant heterogenous nuclear ribonucleoprotein particle protein (hnRNP protein or protein A1) which had not been endogenously methylated (Rajpurohit et al., 1992). Comparing the substrate capability of the two methyl-acceptor proteins, this formerly reported "histone-specific" enzyme actually exhibited one order of magnitude higher affinity toward protein A1 ($K_m = 1.1 \times 10^{-6}$ M) than histone ($K_m = 1.1 \times 10^{-5}$ M) (see Table 3). In the past, however, it was not possible to study an enzyme which methylates arginyl residue of protein A1, because the *in vivo* isolated protein A1 was already stoichiometrically methylated so that it was a very poor methyl-acceptor for *in vitro* study. Due to the recent advances in molecular biology, it is now possible to obtain the recombinant protein A1 through the overexpression of protein A1 gene in *Escherichia coli* vector, which is not posttranslationally methylated (Rajpurohit et al., 1992). Since several other nuclear/nucleolar proteins were also found to contain large amount of N^G-methylarginines (Table 2 and also Lischwe, 1990), further studies are clearly needed to delineate the enzymology of protein methylase I.

Table 3. Comparison of properties of protein methylase I (Ghosh et al., 1988)

Properties	MBP-specific	Histone-specific
M_r (by Sephadex G-200)	500 kDa	275 kDa
Subunit (by SDS-PAGE)	100 kDa, 72 kDa	110 kDa, 75 kDa
pI	5.09	5.68
K_m values:		
MBP	2.3×10^{-7} M	
Histone	1.0×10^{-4} M	1.1×10^{-5} M
Protein A1		1.1×10^{-6} M
S-Adenosyl-L-methionine	4.4×10^{-6} M	8.0×10^{-6} M
K_i values:		
MBP		3.42×10^{-5} M
S-Adenosyl-L-homocysteine	1.8×10^{-6} M	2.30×10^{-6} M
Sinefungin	7.0×10^{-6} M	6.60×10^{-6} M
Dialysis	easily inactivated	not inactivated
50% inactivation:		
p-Chloromercuribenzoate	0.46 mM	0.15 mM
Guanidine.HCl	3.10 mM	0.30 mM
At 50°C for 5 minutes	99% activity remained	60% activity remained

In an analogous situation, protein methylase III [S-Adenosylmethionine: protein-lysine N-methyltransferase; EC 2.1.1.43] has been shown to have several subtypes, each subtype being highly specific towards protein species; cytochrome *c* or cytochrome c_{557}-specific (Nochumson et al., 1977; Valentine and Pettigrew, 1982), calmodulin-specific (Sitaramayya et al., 1980), histone H3 and H4-specific (Sarnow et al., 1981), and ribosomal protein YL23- and YL32-specific (Lobet et al., 1989).

The question of whether one or more than one enzyme is involved in the synthesis of all three N^G-methylarginines (N^G-mono-, N^G, N^G-di-, and N^G, N'^G-di-) remains unclear at present. Uneven distribution of these arginine derivatives (Table 2) suggests that more than a single enzyme is involved. On the other hand, the ratios of the various methylated arginines formed by preparations during the 120-fold enzyme purification were found to be quite constant (Lee et al., 1977), strongly suggesting a single enzyme to be responsible for the formation of three arginine derivatives. This contention was further supported by the product analysis during the time course of wheat germ protein methylase I (Gupta et al., 1982). The formation of both N^G-monomethylarginine and N^G, N'^G-dimethylarginine (sym.) increased up to about 60 minutes of incubation and then reached a plateau. Interestingly, when the percentage of formation of the two methylarginines was plotted as a function of the incubation time, there appeared to be a precursor-product relationship; that is, at early times the yield of N^G-monomethylarginine decreased while that of N^G, N'^G-dimethylarginine increased.

The relative formation of N^G, N^G-dimethylarginine (asym) and N^G, N'^G-dimethylarginine (sym) are greatly dependent on the structure of the protein itself as well as on the interaction with the surrounding structure. Recently, we observed that the amount of N^G-mono- and N^G, N'^G-dimethylarginine (sym) in

myelin basic protein (MBP) increased as a function of the age of the mouse brain whereas that of N^G, N^G-dimethylarginine (asym) decreased (Rawal et al., 1992). MBP from early myelinating mouse brain was shown to contain a high proportion of asymmetric dimethylarginine which was hardly detectable in older brain MBP. This derivative was also absent in MBP embedded in the least compact myelin. In addition, MBPs isolated from dysmyelinating mutant mouse brains such as *jimpy* (jp/y) and *quaking* (qk/qk) contained much higher level of asymmetric N^G, N^G-dimethylarginine relative to the other two arginine derivatives.

Structural requirement of the substrate

All the arginine methylation sites reported to date have at least one adjacent glycine residue. Some of them are flanked by glycine residue and others are found in clusters of glycine residues (Lischwe et al., 1985; Rawal et al., 1992a). When crude rabbit brain protein methylase I was incubated *in vitro* with human MBP, only a single arginine residue at position 107 among a total 18 arginine residues was methylated (Baldwin and Carnegie, 1971). The minimum chain length of MBP polypeptide which functions as the methyl-acceptor has been studied with several synthetic polypeptides whose sequences are identical with the region surrounding the residue 107 of bovine MBP (Ghosh et al., 1990). It was found that the hexapeptide, Gly-Lys-Gly-Arg-Gly-Leu (corresponding to residues 104–109 of bovine MBP) was the shortest methyl-accepting peptide, while the pentapeptide nor tetrapeptide did not serve as the methyl-acceptor. Interestingly, the methylation products of these short peptides by purified MBP-specific protein methylase I and S-adenosyl-L-[*methyl*-^{4}C]methionine yielded only N^G-monomethylarginine. Furthermore, when the N-terminal side of Gly next to the methyl-accepting Arg hexapeptide was substituted with either aspartic acid, phenylalanine, histidine or leucine, these substituted hexapeptides were very poor methyl-acceptor (Ghosh et al., 1990), further confirming the essentiality of the adjacent Gly for the methylation.

Kinetic mechanism

The kinetic mechanism of the protein methylase I reaction was found to be a Sequential Ordered Bi Bi mechanism with S-adenosyl-L-methionine as the first substrate, substrate protein as the second substrate, methylated protein as the first product, and S-adenosyl-L-homocysteine as the second product released (Lee et al., 1977). This is in contrast to a random mechanism for protein methylase II [S-Adenosylmethionine:protein-carboxyl O-methyltransferase; EC 2.1.1.24] in which the rate-limiting step was the interconversion of the ternary complex, enzyme-substrate-adenosylmethionine (Jamaluddin et al., 1975).

Biological significance

Protein-arginine methylation

Currently, there is no experimental evidence to indicate that the guanidino-N of *free* arginine is enzymatically *in vivo* methylated. Since transmethylation

reaction is energy-dependent in the form of S-adenosyl-L-methionine and is catalyzed by a group of highly specific enzymes, it is quite logical to assume that the enzymatic methylation of protein-bound arginine residues play an important role in the regulation of the function and/or metabolism of the protein. Unfortunately, however, biological function of the protein-arginine methylation is not well understood. In the following paragraphs, we will describe some of the better elucidated biological systems of protein-arginine methylation.

Protein-arginine methylation and cell growth

As shown in Table 2, the presence of N^G-methylarginines in histones was first demonstrated by the [*methyl*-^{14}C] incorporation into this basic protein *in vitro* (Paik and Kim, 1967). Subsequently, trace amounts of N^G-methylarginines were detected in histones isolated from sea urchin embryo, however, amino acid sequence analysis of histones from a wide variety of sources, including fish, mammals, insects and plants, have failed to find measurable amounts of N^G-methylarginines (Hnilica, 1972; also cited in Duerre et al., 1991). Possibly due to this lack of *in vivo* methylation, histones have been employed as excellent substrates for the enzyme assays *in vitro*.

When determined with histones as *in vitro* substrates, protein methylase I activity parallels closely the degree of cell proliferation. The enzyme activity was highly elevated in regenerating rat liver, rapidly growing hepatomas, fetal brain and continuously dividing HeLa cell culture (Paik and Kim, 1980b). On closer examination during hepatic regeneration, the *in vivo* [*methyl*-^{14}C] incorporation into histone-arginine residues preceded that of incorporation into histone-lysine residues by 24 hours, and the peak of methylarginine synthesis coincided with that of DNA synthesis in continuously dividing HeLa cell culture.

Many models have been proposed for the alignment of the histones along the major or minor groove of the DNA. To date, none of these models is more supportive than the other. However, one of the more consistent features is that the N-terminal sequence of all the core histones lie outside the nucleosome. The N-terminal sequence of the arginine-rich histones, H3 and H4, appear to be the most accessible to protease digestion (Bohm and Crane-Robinson, 1984). Interestingly, all of the major *in vivo* methylation (ε-N-lysine), acetylation, and phosphorylation sites in the core histones are located in the N-terminal regions (Bohm and Crane-Robinson, 1984). Hence, one or more of these reactions could provide a means by which DNA packaging, accessibility, and/or nucleosome phasing are modulated, thereby influencing gene expression or replication. Indeed, recent observation that arginine residues in histone H2B of *Drosophila* are methylated following heat or chemical shock might suggest such a modulation (Desrosiers and Tanguay, 1988).

The readers should at this point be cautioned of the fact that histone-specific protein methylase I could be identical with protein A1-specific protein methylase I (see section on *Multiplicity of the protein methylase I above*). In addition to its selective binding to single stranded conformation of RNA and DNA (Kumar et al., 1986), protein A1 is known to stimulate α DNA polymerase (Herrick and Alberts, 1976) and to be involved in the splicing and processing of the mRNA

during mRNA biogenesis (Choi et al., 1986). hnRNP proteins (protein A1 is one of them) are located in the nucleoplasm and they are dispersed throughout the cell during mitosis (Pinol-Roma et al., 1989; Choi and Dreyfuss, 1984). Among the different proteins that are transported back into the daughter cell nuclei, protein A1 seems to depend on a transcription-dependent factor for its transport. Thus, it remains to be seen whether methylation of protein A1 in any way influence its various functions or possibly facilitate its transport across the nuclear membrane.

Myelin basic protein (MBP)-arginine methylation

Occurrence of the methylated arginine in MBP was initially observed during the amino acid sequence analysis of this protein (Baldwin and Carnegie, 1971), and an involvement of biological methylation in the integrity and maintenance of myelin has been suggested through the animal experiments which produced subacute combined degeneration (SCD) (Dinn et al., 1980; Scott et al., 1981). Cycloleucine, an inhibitor for S-adenosyl-L-methionine biogenesis, could produce a neurological syndrom similar to vitamin B_{12} deficiency and inhibited methylation of MBP in the brain (Crang and Jacobson, 1980), suggesting that inhibition of MBP methylation might be responsible for the neurological condition. In support of this contention, the MBP-specific protein methylase I activity decreased drastically in dysmyelinating mutant mouse brain during myelinating period (Kim et al., 1984). The mechanism by which MBP participates in the formation of multilayered myelin is not well understood at present, consequently obscuring the significance of MBP-arginine methylation. Notwithstanding, an enzymatic methylation of the critical regions of the myelin molecule may have a significant impact on the molecular organization, as clinical experiments have also indicated an importance of transmethylation reaction in normal nerve function (Scott et al., 1981; Kim et al., 1990).

Effect of methyl substitution on the isoelectric point (pI) of the protein

Methyl substitution of the hydrogen atom on the guanidino group of *free* L-arginine increases its pI values, making the amino acid more basic (Paik et al., 1983a).

	Arg	< N^G-Mono-	< N^G,N^G-Di-	< N^G,N'^G-Di-
pI:	10.02	10.54	10.77	11.01

Contrary to the above, however, when methylation of arginine residue of a protein molecule occurred, an opposite consequence was observed. For example, pI value of horse heart cytochrome *c* decreased by 0.7 pH unit (from 10.03 to 9.33) on enzymatic methylation of Res-38 arginine residue (Farooqui et al., 1985). Similarly, enzymatic methylation of Res-194 arginine residue of protein A1 decreased the pI value by 0.07 (from 9.48 to 9.41) (Rajpurohit et al., 1992). This unexpected effect of methyl substitution of the side chain of protein molecule on its pI value was interpreted to mean that the substitution with CH_3 group can

induce a "global" change on the protein molecule, the effect not just being confined to the immediate surrounding (Paik and Kim, 1992).

Resistance to proteolytic enzyme attack

Analogous to the methyl substitution of the ε-NH_2 group of lysine residues in a protein molecule (Paik and Kim, 1980), methylation of arginine side chains of a protein has often been suggested to protect the protein from intracellular proteolytic enzyme attack (Baldwin and Carnegie, 1971). However, there is no direct evidence to support this contention. Since one of the substrate specificity requirements of trypsin lies on the arginine residue that donates its carbonyl group to the peptide bond to be hydrolyzed, one should distinguish two distinct possibilities; tryptic hydrolysis of peptide bonds whose carbonyl groups are donated by either unsubstituted or methyl substituted arginine. Alternatively, however, one should be aware of the possibility that a methyl substitution could bring about a change in the tertiary structure which allows the protein to adopt a less susceptible configuration (Paik and Kim, 1992).

Metabolism

The amounts in the body fluids

Information concerning the intracellular concentration of free N^G-methylarginines is not available. On the other hand, a few reports on the amounts of these amino acids in blood and urine are listed in Table 4. The amounts of N^G, N^G-dimethylarginine (asym) and N^G, N'^G-dimethylarginine (sym) were significantly elevated in the urine of children with muscular dystrophy (Lou, 1979). The increase of asymmetric dimethylarginine was much greater than that of symmetric form; the ratio of asym to sym isomer was approximately 2.6. In liver diseases, on the other hand, only a slight elevation of asym/sym ratio was observed (Carnegie et al., 1977): 1.31 in patients as compared to 1.08 in normal adults.

The concentration of N^G, N'^G-dimethylarginine in rabbit blood plasma is much less than that of N^G, N^G-dimethylarginine (Table 4). However,

Table 4. Amount of methylated arginines in blood and urine (Kakimoto and Akazawa, 1970; McDermott, 1976)

	Human		Rabbit	
Methylated arginines	serum	urine	plasma	urine
Arginine	54.8*	19–54**	246 ± 23*	10 ± 2**
N^G-Monomethyl	–	–	0.4 ± 0.2	0.1 ± 0.05
N^G, N^G-Dimethyl	0.3	27–64	1.6 ± 0.5	0.1 ± 0.01
N^G, N'^G-Dimethyl	0.3	31–63	0.3 ± 0.1	3.1 ± 0.2

* Expressed as nmoles/per milliliter of serum or plasma
** μMoles/g of creatinine.

McDermott (1976) observed that the urinary excretion of the former is about 30 times greater than that of the latter. This difference, however, does not seem to be caused by a lower renal clearance of N^G, N^G-dimethylarginine compared to that of the other isomer.

N^G-Monomethyl-L-arginine

Among the various methylarginines, this amino acid is the most extensively metabolized *in vivo*. *Escherichia coli* is one of the best known rich source of various amino acid decarboxylases. To maximize the enzyme yield, the bacteria are grown generally in the presence of a particular amino acid to induce the decarboxylase which is specific for the amino acid present. In general, however, these decarboxylases are unstable; thus all the commercially available enzyme preparations are only partly purified. When commercially obtained *E. coli* ornithine decarboxylase preparation (Sigma Chemical Co.) was incubated with N^G-monomethyl-L-arginine, a decarboxylation product N^G-methylagmatine was formed at the rate of 0.36 μmoles/min/mg enzyme protein while 0.20 μmole of L-ornithine was decarboxylated/min/mg enzyme protein (Paik et al., 1981). It is not certain whether this high rate of decarboxylation of N^G-monomethyl-L-arginine was caused by the action of ornithine decarboxylase [EC 4.1.1.17] or due to a contaminating arginine decarboxylase [EC 4.1.1.19]. The decarboxylation product N^G-methylagmatine occurs naturally in seeds of leguminous plants (Matsuzaki et al., 1990).

While investigating the *in vivo* routes of elimination of various methylated arginines in rabbits, McDermott (1976) observed that the urinary excretion of N^G, N'^G-dimethylarginine (sym) was 30 times greater than that of either N^G-monomethylarginine or N^G, N^G-dimethylarginine (asym) (Table 4). The recoveries of intravenously injected amino acids in the urine were 0.14% (N^G-monomethylarginine), 5.1% (N^G, N^G-dimethylarginine), and 66% (N^G, N'^G-dimethylarginine). This result clearly suggested that N^G, N'^G-dimethylarginine (sym) is largely excreted. Thus, McDermott proceeded to investigate the *in vitro* catabolism of N^G-monomethyl-L-arginine with tissue homogenate and found that the kidney contained a high catabolic activity of N^G-monomethyl-L-arginine, which was much higher than that of arginine. Subsequently, we observed that rat kidney contained an enzyme which hydrolyzed N^G-monomethyl-L-arginine to give rise to the formation of L-ornithine and N-methylurea (Paik et al., 1983), suggesting the enzyme to be an arginase-type. However, the kidney enzyme was quite different from the hepatic arginase; commercial bovine hepatic arginase [EC 3.5.3.1] was completely inactive toward N^G-monomethyl-L-arginine.

McDermott (1976) also examined the L-arginine: glycine amidinotransferase [EC 2.1.4.1] activity in various tissues with N^G-monomethyl-L-arginine and L-arginine as substrate. This enzyme is the first of two enzymes to synthesize creatine from L-arginine, glycine and S-adenosyl-L-methionine. N^G-Monomethyl-L-arginine was metabolized at substantial rates in both kidney and liver, and its rate of substrate capability for the enzyme in brain exceeded

that of L-arginine. Although the reaction product has not been identified, the putative product is isocreatine in which the methyl group is located at guanidino nitrogen instead of nitrogen donated by glycine. In spite of this possible *in vivo* synthesis, natural occurrence of isocreatine has not yet been documented.

N^G-Monomethyl-L-arginine could also be degraded by a deiminase, yielding L-citrulline and methylamine. During the studies on the inhibitory effect of N^G-monomethyl-L-arginine on the formation of nitric oxide (NO) from L-arginine in endothelial cells, Hecker et al. (1990) observed that N^G-monomethyl-L-arginine was a less potent inhibitor than N^ω-nitro-L-arginine. This was due to the fact that N^G-monomethyl-L-arginine was metabolized in this tissue by an enzyme with the characteristics of a deiminase, N^G, N^G-dimethylarginine dimethylaminohydrolase (Osawa et al., 1989) (see below).

In conclusion, N^G-monomethyl-L-arginine could be degraded *in vivo* by several metabolic routes, including decarboxylation, hydrolysis by an arginase-type enzyme, transfer of methylamidine to glycine, and deimination by a novel enzyme which has recently been identified. This is the most likely explanation why this amino acid is hardly detected in human blood and urine (Table 4).

Before going to the next subject, we would like to speculate one more involvement of N^G-monomethyl-L-arginine in the formation of methylguanidine. Methylguanidine, which is a normal constituent of human urine and blood and is considered to be a toxin in uremia, has been suggested to be derived from creatine. However, knowledge on the identity of the enzyme involved as well as the mechanism remains to be elucidated (Yokozawa et al., 1991). On examining the structure of both creatine and N^G-monomethyl-L-arginine, one finds a great similarity among them; the former is *methylamidino*glycine and the latter *methylamidino*ornithine. Thus, it is suggested that N^G-monomethyl-L-arginine could also be a good candidate for the synthesis of methylguanidine.

N^G, N^G-Dimethyl-L-arginine (asymmetric)

When N^G, N^G-dimethyl-L-[1,2,3,4,5-^{14}C]arginine was injected to rats, approximately 13% of the radioactivity was recovered in the urine during the first 12 hours and the radioactivity was found in the following metabolites: unchanged N^G, N^G-dimethyl-L-arginine, γ-(N,N-dimethylguanidino)butyric acid, α-keto-δ-(N,N-dimethylguanidino)valeric acid and N^α-acetyl-N^G, N^G-dimethyl-L-arginine in decreasing order (Ogawa et al., 1987). The radioactivity remaining in the tissues was mainly associated with citrulline, ornithine, arginine, glutamic acid and protein-bound arginine. This result suggests that N^G, N^G-dimethyl-L-arginine was metabolized in rat tissues by at least three pathways. The first metabolic pathway is the direct deamination (either by oxidative deamination or transamination) and subsequent decarboxylation of the formed α-ketoacid. The second route is the acetylation of the α-amino group of the amino acid. Finally, N^G, N^G-dimethyl-L-arginine was deiminated to form citrulline as in the case of N^G-monomethyl-L-arginine. Dimethylamine is the other reaction product.

Ogawa et al. (1989) subsequently purified an enzyme (N^G, N^G-dimethylarginine dimethylaminohydrolase) from rat kidney which catalyzed the

formation of L-citrulline and dimethylamine from N^G, N^G-dimethyl-L-arginine. Highly purified enzyme (830-fold) was very specific toward N^G, N^G-dimethyl-L-arginine with a K_m value of 0.18 mM and V_{max} of 0.28 units/mg enzyme protein. Although N^G-monomethyl-L-arginine served as a less efficient substrate (K_m of 0.36 mM and V_{max} of 0.17 unit/mg protein), N^G, N'^G-dimethyl-L-arginine (sym) was completely inert as a substrate.

As for the formation of α-keto derivative of N^G, N^G-dimethyl-L-arginine, Ogawa et al. (1990) recently purified an enzyme (Dimethyl-arginine:pyruvate aminotransferase) to homogeneity from rat kidney. The enzyme catalyzed the transamination of N^G, N^G-di- and N^G, N'^G-dimethyl-L-arginine to pyruvate or glyoxylate. However, the physicochemical and immunological analysis of the purified enzyme showed that the enzyme was identical with one of the isozymes of alanine: glyoxylate aminotransferase [EC 2.6.1.44].

N^G, N'^G-Dimethyl-L-arginine (symmetric)

Similar to the asymmetric isomer discussed above, Ogawa et al. (1987) injected N^G, N'^G-dimethyl-L-[1,2,3,4,5-^{14}C]arginine into rats, and approximately 75% of the radioactivity was recovered in the urine in the first 12 hours. Relative ratios of 24% of the urinary radioactivity was accounted for unchanged N^G, N'^G-dimethyl-L-arginine, 48% for N^α-acetyl-N^G, N'^G-dimethyl-L-arginine, 20% for α-keto-δ-(N,N′-dimethylguanidino)valeric acid and 10% for γ-(N,N′-dimethylguanidino)butyric acid. In the tissues, most of the radioactivity was associated with unchanged N^G, N'^G-dimethyl-L-arginine. This finding suggests that N^G, N'^G-dimethyl-L-arginine was deaminated and acetylated to a much less extent than with N^G, N^G-dimethyl-L-arginine. Deimination of the compound did not occur: This *in vivo* observation has been borne out *in vitro* enzyme assay, as mentioned earlier.

Pharmacological effect

A very interesting, but not-yet confirmed, observation on the growth-retarding effect of N^G-monomethyl-L-arginine has been made (Tyihak and Patthy, 1973). It is known that there exists an *in vivo* antagonism between lysine and arginine (Roess and De Busk, 1968; Jones et al., 1967; Szende and Tyihak, 1968). For example, either oral or peritoneal administration of a large dosage of L-lysine strongly inhibited tumor-growth in an animal, whereas L-arginine promoted the growth of the tumor. On the other hand, corresponding antipods resulted in an inverse effect on tumor growth; L-lysine promoted the tumor growth, whereas D-arginine retarded it. In contrast to L-lysine and L-arginine, ε-N-trimethyl-L-lysine has been found to stimulate Ehrlich and NK/Ly ascites tumor cell growth (Kopper et al., 1971) along with plastic transformation of a proportion of the tumor-dependent lymphocytes (Tyihak et al., 1990). On the other hand, N^G-monomethyl-L-arginine retarded the growth of tobacco tissue culture; N^G-monomethyl-L-arginine inhibited the weight growth by 40% at the concentration of 10 mg/liter of culture medium (Tyihak et al., 1990).

Recently, Hibbs and his coworkers (Hibbs et al., 1987; and 1987a) observed that the antitumor activity of macrophage was due to the metabolism of L-arginine to citrulline, nitrate and nitrite. Subsequent studies established that nitrate and nitrite are derived from nitric oxide (NO), which is in turn derived from the guanidino group of L-arginine (Palmer et al., 1988). Since N^G-monomethyl-L-arginine inhibits NO synthesis in endothelial cells as well as activated macrophages (Hibbs et al., 1987; Rees et al., 1989), the growth-retarding effect of N^G-monomethyl-L-arginine in tobacco tissue culture (Tyihak et al., 1990) cannot be explained merely on the basis of NO synthesis: Via NO, N^G-monomethyl-L-arginine is expected to stimulate the growth in tissue culture.

Inhibitory effect of N^G-monomethyl-L-arginine on nitric oxide (NO) synthesis

Nitric oxide is synthesized in macrophages, neutrophils, endothelial cells, cerebellum and hepatocytes. The presence of NO has been shown to be involved in several important biological events including vascular smooth muscle relaxation, platelet deaggregation, neuronal communication and photoreceptor signaling (Moncada and Higgs, 1990). Earlier in this endeavour, it has been recognized that N^G-monomethyl-L-arginine inhibits the NO synthesis (Hibbs et al., 1987; Rees et al., 1989), and Hibbs et al. (1987) proposed that this methylated amino acid most likely inhibited arginine deiminase [EC 3.5.3.6]. This enzyme is quite different from the enzyme N^G, N^G, dimethylarginine dimethylaminohydrolase identified by Ogawa et al. (1989); the enzyme of Ogawa et al. did not hydrolyze L-arginine at all.

Finally, a recent application of N^G-monomethyl-L-arginine in alleviation of clinical symptoms should be mentioned. Septic shock is a life-threatening condition that results from exposure to bacterial endotoxin. Kilbourn et al. (1990) injected bacterial endotoxin (40 μg/kg body weight) into dog and observed a 33% decrease in peripheral vascular resistance and a 54% fall in mean arterial blood pressure within 30 to 90 minutes. On intravenous administration of N^G-monomethyl-L-arginine (20 mg/kg of body weight), vascular resistance and systemic arterial pressure returned to normal within 1.5 minutes. Therefore, the utility of NO synthesis inhibitor suggests that N^G-monomethyl-L-arginine may be of therapeutic value in the treatment of septic shock.

Concluding remarks

N^G-Methylarginines are posttranslationally synthesized *in vivo* with S-adenosyl-L-methionine as the methyl donor. Three major N^G-methylarginines (N^G-mono-, N^G, N^G-di- and N^G, N'^G-di) are found ubiquitously among prokaryotic organisms and their synthesis are catalyzed by a group of enzymes called protein methylase I. These enzymes are highly specific for arginine residues as well as protein species. Thus, identities of two enzymes (MBP- and protein A1-specific) have been clearly established and there are strong indication that histone H4-specific enzyme in wheat germ and cytochrome *c*-specific in *Euglena gracilis* also exist. More protein species-specific protein methylase I are expected to be discovered. It is concluded that quite moderate advance has been made in the

understanding of the enzymology involved in protein-arginine methylation. On the other hand, biochemical function of the reaction is not well appreciated, even though we can claim some inroad in the study of MBP-arginine methylation during myelination process.

The most important recent development in protein-arginine methylation research is the finding that N^G-monomethyl-L-arginine acts as an antagonist to NO synthesis which attracted a great deal of attention in recent years. Since N^G-methylarginines have been shown to be growth-retardants in tissue culture systems, a question arises on the mechanism of growth-retarding effect. Recently, N^G-monomethyl-L-arginine was utilized in alleviating septic shock in dog. It is hoped that more research affort should be paid to the understanding of the pharmacological effect of N^G-methylarginines. Related to this, information on the intracellular concentrations of N^G-methylarginines in normal and diseased conditions may allow one to understand more insight of protein-arginine methylation.

N^G-Methyl-L-arginines are further metabolized by several pathways; decarboxylation, transamination, acetylation and deimination. For example, deimination of N^G, N^G-dimethyl-L-arginine is catalyzed by an highly specific enzyme in rat kidney, and N^G-monomethyl-L-arginine is hydrolyzed or decarboxylated by highly specific enzymes in rat kidney and *Escherichia coli*, respectively. Since N^G-methyl-L-arginines constitute very minor portion of the free amino acids in tissue, a question arises why such array of specific enzymes are required for the seemingly "unimportant" amino acids. What is the biochemical significance of N-methylagmatine in *Legminous*, and high concentrations of N^G-methyl-L-arginines in fungi?

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