Localization of Methylated Arginine in the A1 Protein from Myelin

STEVEN BROSTOFF AND E. H. EYLLAR*

The Salk Institute, San Diego, California 92121

Communicated by Max Tishler, January 28, 1971

ABSTRACT Methylated arginine residues are found at only one site (position 107) of the polypeptide chain of the A1 protein, as shown by analysis of tryptic and peptic peptides; these analyses show 0.2 mole of N\textsuperscript{0}-dimethylarginine and 0.4-0.8 mole of N\textsuperscript{0}-monomethylarginine per mole of A1 protein. The methylated arginine residues appeared to be relatively resistant to tryptic attack. Both methylated derivatives were isolated from an enzymatic digest of the A1 protein; they were identified by chromatography, electrophoresis, and degradation to citrulline, methylamine, and ornithine on alkaline hydrolysis. The phylogenetic importance of the methylated derivatives was shown by their presence in the human, monkey, bovine, rabbit, guinea pig, rat, chicken, and turtle A1 proteins at the analogous position to that of the bovine sequence:

(Methyl)\_ (x = 1 or 2)\n
-Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg-107

We postulate that the methylated arginine residues may serve an important role in the myelin membrane in situ by stabilization of a double-chain structure for the A1 protein; such a double-chain conformation is induced by a (proline) sequence located nearby.

Modification of amino acid residues in proteins, while rare, has been encountered in the basic residues, lysine and histidine. N\textsuperscript{-}Methyllysine was found in bacterial flagellin (1), 3-N-methylhistidine was found in actin (2), and N\textsuperscript{-}methyllysine and an N\textsuperscript{-}acetylylsine have been located at specific positions of the amino acid sequence of histone IV (3). Enzymes were partially purified from thymus and brain that methylated arginine residues of endogenous protein acceptor (4) and exogenous acceptors, including calf-thymus histones (5). These data, together with the finding of "ω-N-methylarginine" in various histones (6) and two different methylated arginines in urine (7), provide significant evidence for the methylation of arginine in histones.

We report here the first localization of methylated arginine residues at a specific position of the polypeptide chain of a nonhistone protein, the A1 protein of bovine spinal-cord myelin. Both N\textsuperscript{0}-monomethylarginine and N\textsuperscript{0}-dimethylarginine appear at position 107 of the recently reported sequence of A1 protein (8).

EXPERIMENTAL PROCEDURE

Hydrolysis of the A1 protein

Preparation of the A1 protein from bovine spinal cord, and brains of other species (9, 10), has been described (11). A1 pro-tein (3.2 g) was incubated at 37°C, pH 8.0, with 150 mg of pronase and 0.1 ml of toluene. An additional 150 mg of pronase was added after 24 hr. After 46 hr, the solution was boiled for 10 min, filtered, and then incubated with 10 mg of amino peptidase M for an additional 18 hr at 37°C, pH 8.2, and lyophilized.

Column chromatography of protein hydrolysate

A column of Dowex 50 W \texttimes 4 (2.6 \texttimes 60 cm) was equilibrated with pyridine-acetate buffer, pH 3.1 (12). The enzymic digest of the A1 protein, containing [\textsuperscript{14}C]phenylalanine and [\textsuperscript{3}H]-arginine as markers, was dissolved in 150 ml of this buffer, adjusted to pH 2.1, and applied to the column. A gradient was established by adding 1.0 liter of pyridine-acetate buffer, pH 5.0 (12), to a closed reservoir containing 300 ml of pyridine-acetate buffer, pH 3.1 (tubes 1–140). The column was then eluted successively with 1.5 liter of 2 N pyridine (tubes 141–210), 1.0 liter of 0.5 N NH\textsubscript{4}OH (tubes 211–310), and 1.0 liter of 3 N NH\textsubscript{3}OH (tubes 311–400). The flow rate was 65 ml/hr; 10-ml fractions were collected. The eluate was monitored by pH, radioactivity, and ninhydrin assay (13).

Methods

Ascending chromatography was for 18 hr on Whatman 3 MM paper in a solvent (7) of pyridine-acetone-3 M NH\textsubscript{4}OH 50:30:25. Paper electrophoresis was in 0.05 N formic acid, pH 2.7, at 2.5 kV for 90 min. Peptide mapping of the tryptic peptides from the A1 protein has been described (11). Tryptic and peptic peptides were sequenced by the direct and indirect Edman procedures (14).

Weak alkaline hydrolysis was performed in 0.1 N NaOH for 3 hr at 95°C under N\textsubscript{2}. Strong alkaline hydrolysis was performed in a suspension containing 600 mg/ml of Ba(OH)\textsubscript{2}.8 H\textsubscript{2}O under N\textsubscript{2} at 125°C for 20 hr. Acid hydrolysis was in 6 N H\textsubscript{2}SO\textsubscript{4} for 24 hr at 110°C under N\textsubscript{2}.

Amino acids were analyzed on the Beckman autoanalyzer (11). In our hands, lysine and ornithine were eluted from the short basic column at 22 min, histidine at 25 min, NH\textsubscript{4} at 30 min, methylamine at 35 min, and arginine at 45 min. Citrulline was eluted from the long column at the same position as proline. Traces of NH\textsubscript{4} were found in all samples.

RESULTS

When the A1 protein was analyzed on the basic column for its amino acid composition, an unusual peak (8) was observed between ammonia and arginine (denoted by arrow, Fig. 1E); methylated arginine residues have previously been observed in this region (6, 7). Although the residue responsible for this
peak constituted only 0.2 mol/mol protein, we decided to isolate and identify this material.

Bovine A1 protein (3.2 g) was treated successively with pronoase and aminopeptidase M. The digest, along with [1 C]-phenylalanine and [3H]arginine, was then applied to a Dowex 50 column (not shown). [1 C]Phenylalanine was eluted from the column at pH 3.5, whereas [3H]arginine was eluted much later, in 3 M NH₄OH. Two major ninhydrin-positive peaks appeared, tubes 297–306 and 327–335 (lysine and arginine, respectively). Since the ninhydrin assay on tubes between these peaks indicated the presence of trace components (not shown), this region was surveyed by paper electrophoresis; part of the results are shown in Fig. 2B. In addition to lysine, a slower-moving spot was discovered in tubes 308–322 (see tube 315, Fig. 2B), while tubes 323–332 contained a spot migrating slower than, but partially overlapping with, arginine (see tube 325, Fig. 2B).

Isolation of N⁰-dimethylarginine

On the basis of the paper electrophoretic survey, tubes 308–322 were combined; the two major components of this fraction were separated by preparative paper electrophoresis. The slower band, designated as fraction A, was eluted with 0.5 N acetic acid (17% yield, 1.2 mg), and analyzed on the amino acid analyzer. It had a characteristic time of elution from the basic column of the Beckman analyzer of 40 min: arginine is eluted at 45 min. The elution curve of fraction A, shown in Fig. 1C, contains only one major component; NH₄, and a trace component at the arginine position, are also present. Fraction A showed one component on paper chromatography that moved at the same rate as lysine (Fig. 2A); this Rf has been reported for dimethylarginines in this solvent (7).

Further studies (Table 1) revealed that fraction A was identical with dimethylarginine. It was stable to acid hydrolysis (6 N HCl, 24 hr, 110°C), but strong alkaline hydrolysis gave nearly quantitative conversion to ornithine. Weak alkaline hydrolysis gave citrulline as the main degradation product. We did not determine whether fraction A was the N⁰,N⁰-dimethylarginine or the N⁰,N⁰-dimethylarginine derivative; both modifications could have been present. Methylamine (see Table 1) was always found after alkaline hydrolysis, which shows the presence of the N⁰,N⁰-dimethyl derivative, whereas citrulline is consistent with the presence of the N⁰,N⁰-dimethyl derivative (7).

Isolation of N⁰-monomethylarginine

Tubes 323–332 contained material that migrated only slightly slower than arginine (Fig. 2B). Preparative ascending chromatography was used to separate this material, designated fraction B, from arginine. This fraction had an Rf, with respect to lysine, of 0.69; it separated well from arginine, which has an Rf (relative to lysine) of 0.42 (see Fig. 2A). Fraction B showed a characteristic time of elution from the amino acid analyzer of 44 min, preceding arginine by only 1 min (see Fig. 1D), and thus virtually inseparable under ordinary elution con-
ditions. As seen from Fig. 1D, fraction B, prepared by chromatography, contains only one peak (along with NH₄ and a trace of lysine), and was used for further characterization.

Like fraction A, fraction B was stable to acid hydrolysis; however, under strong alkaline conditions, it was quantitatively converted to ornithine, as shown in Table 1. On mild alkaline hydrolysis, fraction B gave citrulline as the major breakdown product. Methylamine, with an elution time of 35 min, appeared on analyses after both strong and mild alkaline hydrolysis and, with mild hydrolysis, the conversion was nearly quantitative, as based on the sum of the ornithine and citrulline values. These data are all consistent with the identification of fraction B as N₂-monomethylarginine.

**Location of methylated arginine in the A1 protein**

Of all the tryptic and peptic peptides isolated during the determination of the sequence of the A1 protein (15), only 2 peptides were found that contained dimethylarginine: tryptic peptide T16A and peptic peptide P10A. From the sequences of these peptides, shown in Table 2, it can be seen that the arginine residue 107 is common to both peptides, which establishes this position as the only focus for methylation in the A1 protein. It should be noted that peptic peptides P10 and P10A have the identical sequence, and differ only at position 107 which, in peptide P10A, is completely accounted for as the dimethylarginine derivative. Apparently it is this slight difference that accounts for the separation of these two peptides on Cellex-P chromatography (15).

It is obvious that tryptic peptide T17 (see Table 2) is derived from tryptic peptide T16A by cleavage of the Arg-Gly linkage located between positions 107 and 108. In peptide maps of the tryptic digest of the A1 protein, these two peptides were always found, generally in a 60:40 ratio of peptide T16A to T17. It is apparent, therefore, that the arginine residue at position 107 is partially resistant to trypsin attack, presumably because of methylation. Only peptide T16A contained dimethylarginine; the dimethyl derivative accounted for 30% of the residues at this position (see Table 2). In view of its resistance to trypsin, the remainder of the arginine at position 107 of peptide T16A must be the monomethyl derivative.

The relative proportion of methylated arginine residues at position 107 of the A1 protein can therefore be estimated. Since peptides T16A and T17 are derived in a 3:2 ratio, and the dimethylarginine derivative accounts for 30% of the unhydrolyzed arginyl bond of peptide T16A, this derivative must comprise approximately 0.2 mol/mol of A1 basic protein, a value in good agreement with the amount of dimethylarginine obtained directly from the complete hydrolysis of the A1 protein (as shown in Fig. 1E). Thus, all of the dimethylarginine of the A1 basic protein, 0.2 mol/mol, is present at position 107. The remainder of the arginine of peptide T16A appears to be the monomethylarginine derivative and would account, therefore, for 0.4 mol/mol of protein. Thus, a total of 0.6 mol of methylated arginine derivatives is present at position 107. If we assume that the monomethylarginine is completely resistant to trypsin, then the 0.4 mol of monomethylarginine represents a maximum value. However, the peptide linkage involving the monomethylarginine may be slowly hydrolyzed by trypsin. In that case, all of the arginine at position 107 could be methylated, i.e., 0.8 mol of monomethylarginine and 0.2 mol of dimethylarginine per mol of A1 protein.

**Phylogenetic distribution of dimethylarginine**

In order to determine the presence and location of dimethylarginine in A1 proteins from various animal species, the pep-

---

**Table 1. Products derived from fractions A and B after alkaline and acid hydrolysis**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fraction A</th>
<th>Fraction B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong acid hydrolysis (6 N HCl, 110°C, 24 hr)</td>
<td>Fraction A (100%)</td>
<td>Fraction B (100%)</td>
</tr>
<tr>
<td>Strong alkaline hydrolysis (600 mg/ml Ba(OH)₂·8 H₂O, 125°C, 20 hr)</td>
<td>Ornithine (84%)</td>
<td>Ornithine (100%)</td>
</tr>
<tr>
<td></td>
<td>Fraction A (16%)</td>
<td>Methylamine</td>
</tr>
<tr>
<td>Weak alkaline hydrolysis (0.1 N NaOH, 95-100°C, 3 hr)</td>
<td>Ornithine (27%)</td>
<td>Ornithine (40%)</td>
</tr>
<tr>
<td></td>
<td>Citrulline (60%)</td>
<td>Citrulline (51%)</td>
</tr>
<tr>
<td></td>
<td>Fraction A (13%)</td>
<td>Fraction B (9%)</td>
</tr>
<tr>
<td></td>
<td>Methylamine†</td>
<td>Methylamine†</td>
</tr>
</tbody>
</table>

* The percentage of each product after alkaline hydrolysis was determined from the total recovery from the amino acid analyzer. For acid hydrolysis, the percentage refers to the recovery from a standard solution. Arginine is quantitatively converted to ornithine under conditions of strong alkaline hydrolysis.

† For fraction A, under conditions of weak alkaline hydrolysis 0.35-0.6 mol of methylamine was found per mol of (ornithine plus citrulline).

‡ For fraction B, a nearly theoretical yield of methylamine was obtained under conditions of weak alkaline hydrolysis: 0.9 mol/mol of (ornithine plus citrulline).

---

**Table 2. Tryptic and peptic peptides isolated from the A1 protein containing the region surrounding residue 107**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>T16A</th>
<th>T17</th>
<th>P10</th>
<th>P10A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg(methyl)₂</td>
<td>0.3 (2)</td>
<td>0.3 (2)</td>
<td>0.9 (1)</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.4 (1)</td>
<td>1.3 (1)</td>
<td>0.9 (1)</td>
<td>1.1 (1)</td>
</tr>
<tr>
<td>Thr</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>2.0 (2)</td>
<td>2.2 (2)</td>
<td>2.0 (2)</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Gly</td>
<td>1.6 (2)</td>
<td>1.2 (1)</td>
<td>3.2 (3)</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Leu</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>1.9 (2)</td>
<td>2.0 (2)</td>
</tr>
</tbody>
</table>

* The sequences of tryptic peptides T16A and T17 and peptic peptides P10 and P10A are:

(Methyl) or S

Peptide T16A: Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg

(107) (110)

Peptide T17: Gly-Leu-Ser-Leu-Ser-Arg

Peptide P10: Pro-Arg-Thr-Pro-Pro-Pro-Ser-Gly-Gly-Arg-Gly-Leu-Ser-Leu

Peptide P10A: Pro-Arg-Thr-Pro-Pro-Pro-Ser-Gly-Gly-Arg-Gly-Leu-Ser-Leu

(107) (110)

Lys: Gly-Arg-Gly-Leu-Ser-Leu

(Methyl)₂
residues of Lys-Asn-Ile protein containing (Methyl).
For the dimethylarginine technique NG-monomethylarginine tryptic chymotryptic peptide, however, appears per arginine in proteins.

The three small arrows point to proline residues of the Pro-Pro-Pro sequence; the larger arrow points to the N°-monomethylarginine side chain.

**DISCUSSION**

The data reveal that position 107 of the A1 protein is occupied by both dimethylarginine (20%) and monomethylarginine (40–80%). What is the significance of the methylated derivatives in the A1 protein, and why are they found at only one position? Methylated arginine residues are very rare in proteins (6); our findings represent the first localization of such derivatives in a specific protein. Moreover, the finding of the dimethylarginine derivative in the A1 proteins from chicken, turtle, and all mammalian species examined reveals the phylogenetic importance of the methylated derivative. Thus, it is reasonable to assume that the methylation may play an important role associated with the structure, the synthesis, or the biological properties of the A1 protein.

The A1 protein comprises 30% of the total protein of myelin (9) and probably contributes to the structural integrity of the myelin membrane by electrostatic and nonpolar interactions with the lipid components (8, 17, 18), a role expedited by the basic properties (8, 17) and highly open conformation of the A1 protein (11, 17). In solution, the A1 protein complexes with negatively-charged proteins and phospholipids (18), particularly phosphoinositides, and appears to be designed for maximal interaction with myelin components. Nothing is known of the conformation of this protein in situ, however, where interactions with lipids, and possibly proteolipid, could induce significant deviation from its unfolded structure in solution. Nonetheless, it appears appropriate to consider the region of the polypeptide chain containing the methylated arginine (position 107), a segment that includes the acceptor sequence (Thr-Pro-Pro-Pro), the target of glycosylation by the N-acetylgalactosaminyl transferase from submaxillary glands (19). The (proline)3 sequence forms a sharp bend in the polypeptide chain, thereby inducing a double-chain structure, as illustrated by the model in Fig. 3. Such a double-chain conformation would be compatible with viscosity studies, where a 10:1 axial ratio was found (17); this figure is lower than that expected for a truly random molecule.

A question arises concerning the relevance of an open, double-chain structure of the A1 protein to the myelin membrane itself. The (proline)3 bend occurs near the midpoint of the A1 molecule, thus setting a length limit for the double chain. Fully extended, it could reach 500 Å, assuming 5 Å per amino acid residue. Such a structure, while providing a backbone for association with lipids, might be stabilized by interchain interaction between compatible side chains. In this re-

**TABLE 3. Amino acid composition of tryptic peptides containing dimethylarginine from various species**

<table>
<thead>
<tr>
<th></th>
<th>Bo-</th>
<th></th>
<th>Guinea</th>
<th>Human</th>
<th>Monkey</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td></td>
<td>1.0†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>(Methyl)3</td>
<td>1.4</td>
<td>1.0</td>
<td>1.3</td>
<td>1.4</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Thr</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>2.0</td>
<td>2.1</td>
<td>1.9</td>
<td>1.9</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Gly</td>
<td>1.6</td>
<td>1.7</td>
<td>1.8</td>
<td>1.8</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Leu</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Turtle also contained dimethylarginine at the same position, 0.2 mol/mol of peptide, as determined from a chymotryptic peptide analogous to peptide P10A.
† Ratios normalized to Leu (2.0).
Methylated Arginine in the Al Protein

...ard, it is tempting to speculate that a structural relationship might exist between the (proline) 3 bend and the methylated arginine, since both are extremely rare in proteins, yet occur in close proximity in the Al protein. The methylarginine could provide one of several sites for cross-chain stabilization of a double-chain conformation, either by interaction with lipid elements or, possibly, in conjunction with the adjacent phenylalanine side chains found in close proximity, as shown in Fig. 3. Such interaction might be promoted by the increase in hydrophobic character of the guanidino group by methylation.

We thank Dr. F. Westall and Dr. P. Carnegie for helpful discussions, and Mr. J. Jackson for expert technical assistance.

This investigation was supported by U.S. Public Health Service grant 1RO1 NB 08268-02.