Determination of Methylated Basic Amino Acids with the Amino Acid Analyzer

APPLICATION TO TOTAL ACID HYDROLYZATES OF MYELIN BASIC PROTEINS*

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SUMMARY

A procedure is described for the direct determination of methyl derivatives of basic amino acids in total acid hydrolyzates of proteins by the use of a Beckman amino acid analyzer operated in the high sensitivity mode with automatic integration. The conditions described permit the separation of ornithine, lysine, 5-hydroxylysine, N'-monomethyllysine, N'-dimethyllysine, histidine, N'-methylhistidine, ammonia, methylvamine, NG,NG-dimethylarginine, N,G-dimethylarginine, N'-monomethylarginine, and arginine. The procedure was used to survey purified myelin basic proteins prepared from a wide variety of species for their contents of methylated basic amino acids. Of the latter only NG,NG-dimethylarginine and NG-monomethylarginine were present. The proportions of the two methylarginines, as well as the fraction of the total arginine which they comprise varied widely depending upon the species source. No NG-monomethylarginine was detected in the hydrolyzate of the frog myelin basic protein; no methylarginines were found in that of the carp. Hydrolyzates of fractions of contaminating proteins which had been removed from the turtle myelin basic protein during its purification were found to contain appreciable quantities of NG,NG-dimethylarginine.

Estimation of the content of NG-monomethylarginine in proteins has been hampered by inadequate separation of this derivative from arginine on the amino acid analyzer (9, 10, 12), even when the two amino acids are present in comparable amounts (9, 10). Brostoff and Eylar (10) inferred the content of NG-monomethylarginine from the dimethylarginine content and the extent to which the peptide bond involving arginine 107 was cleaved by trypsin. Baldwin and Carnegie (9) determined the NG-monomethylarginine to dimethylarginine and (NG-monomethylarginine plus arginine) to dimethylarginine ratios and estimated the relative amount of unsubstituted arginine at position 107 by difference. Both studies involved enzymatic digestion of the protein and isolation of peptides containing arginine 107. In none of these studies were the arginine derivatives compared with authentic methylarginine standards.

The present communication describes a procedure for the direct determination of NG,NG-monomethyl-, NG,NG-dimethyl-, and NG,NG-dimethylarginine in total acid hydrolyzates of proteins on the Beckman amino acid analyzer. The use of a high resolution cation exchange resin permits the determination of all three of the methylarginines as well as ornithine, 5-hydroxylysine, N'-monomethyllysine, N'-dimethyllysine, and N'-methylhistidine. The procedure has been used to determine the contents of methylarginines in myelin basic proteins of several species, both mammalian and submammalian, and the results are compared with those previously reported by others (9-11) by indirect techniques.

MATERIALS AND METHODS

Amino Acid Standards—L-Arginine HCl (NRC), L-ornithine HCl (NRC), 5-hydroxylysine HCl, N'-methyl-L-lysine HCl,
N*-dimethyl-L-lysine HCl, 3-methyl-L-histidine (N*-methylhistidine), and 1-methyl-L-histidine (N*-methylhistidine) were products of Cyclo Chemical Corp., Los Angeles, Calif. Methylamine hydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. Beckman amino acid calibration mixture type 1 was a product of Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif. N5,N5-Dimethyl-, N6,N6-dimethyl- and N5-monomethylarginine were generously provided by Dr. M. Reporter, C. F. Kettering Research Lab., Yellow Springs, Ohio. The methylarginines were prepared by the general method of Kakimoto and Akazawa (13) except that thiourea derivatives, rather than urea derivatives, were used to give higher yields. Analysis of a relatively large quantity of each of the methylarginine standards (arginine equivalents, 0.04 to 0.22 pmole) by the procedure described below revealed ornithine (0.2 to 3.6% of the total impurity detectable other than a small quantity of ammonia. Solutions (2.5 pmoles per ml) of ornithine, 5-hydroxylsine, N*-monomethyllysine, N*-dimethyllysine, N*-methylhistidine, and methylamine were made in 0.01 N HCl; N*-methylhistidine in 0.01 N HCl was present at a concentration of 5.0 pmole per ml. Solutions of the methylarginines were made in deionized water. All solutions were stored at 5°C.

Standard Calibration Mixture—The standard calibration mixture was made by diluting the above solutions of Beckman standard type 1 calibration mixture, ornithine, methylamine, N*-monomethyllysine, and N*-methylhistidine and methylamine were made in 0.01 N HCl; N*-methylhistidine in 0.01 N HCl was present at a concentration of 5.0 pmole per ml. Solutions of the methylarginines were made in deionized water. All solutions were stored at 5°C.

Analysis of Basic Amino Acids and Their Methylated Derivatives—The system used was a modification and combination of several which have been described previously by other workers (14-18). Analyses were carried out with a Beckman 120C amino acid analyzer operated at high sensitivity. Peak integration was performed by an Instrumental CRIS 110A integrator with digital printout. Amounts of amino acids up to 1 pmole could be accurately quantitated; however, resolution permitting accurate quantitation of N5-monomethylarginine in protein hydrolysates was generally obtained with samples containing 0.54 pmole or less of arginine. When larger samples of hydrolysates were examined either in attempts to detect traces of some of the methylated basic amino acids or to quantitate ornithine accurately, N5-monomethylarginine and arginine were often insufficiently separated for accurate quantitation. In such cases approximate integration of the partially resolved N5-monomethylarginine peak was carried out manually. All samples were applied to the columns with Beckman manual sample injectors having a 250-μl sampling loop.

Chromatography was carried out on a column (0.9 x 30 cm) of DC-2A resin (Durom Chemical Corp., Palo Alto, Calif.). The starting buffer and temperature were 0.65 N sodium citrate, pH 5.80 or 5.84, and 28°C, respectively. At 200 min the buffer was changed to 0.25 N sodium citrate, pH 4.70, and the temperature was increased to 55.5°C. The resulting change in absorbance of the eluent occurred at a time when no amino acids were being eluted. The flow rate was 45 ml per hour with initial back pressures of 300 p.s.i. (28°C) and 200 p.s.i. (55.5°C). After every second analysis the column was automatically regenerated with 0.2 N NaOH at a flow rate of 45 ml per hour (55.5°C) for 15 min. The column was then equilibrated with pH 5.80 or 5.84 buffer at 55.5°C for 35 min, and the temperature was subsequently lowered to 28°C.

Proteins—Analyses described in this communication were carried out on the following samples of protein, lyophilized, and stored desiccated at 5°C: (a) guinea pig basic protein prepared in 1961 from purified myelin as described by Laatsch et al. (19); (b) bovine, rabbit, human, and monkey myelin basic proteins extracted at pH 3.0 from delipidated brain or spinal cord tissue and prepared as described previously by a large scale procedure (20) with further purification achieved by a single passage through Sephadex G-150 in 0.01 N HCl at 5°C; (c) guinea pig, rat,1 bovine, frog, and carp myelin basic proteins extracted at pH 3.0 from delipidated spinal cord tissue and purified solely by gel filtration as described above; (d) turtle myelin basic protein similarly extracted from delipidated brain and purified by two passages through Sephadex G-150, followed by chromatography on carboxymethylcellulose at 5°C in 2 M urea at pH 11 with a linear gradient of NaCl (23). Four chromatographic fractions were obtained: unadsorbed proteins, tubes 7 to 14; two fractions of moderately basic protein, tubes 15 to 29 and 30 to 40, eluted at the beginning of the NaCl gradient; and the highly basic myelin protein, tubes 62 to 122. The first three fractions were found to be free of myelin basic protein as judged by polyacrylamide gel electrophoresis at acid and alkaline pH (20). Characterization of the turtle, frog, and carp myelin basic proteins as well as details of the chromatographic purification of the turtle myelin basic protein will be presented elsewhere.2 The central nervous tissues used as sources of the myelin basic proteins were quick frozen in liquid nitrogen or Dry Ice as soon as possible post mortem. In addition to the above proteins prepared in our laboratory, a sample of bovine myelin basic “A1” protein (24) kindly provided by Dr. E. H. Eylar, Merck Institute, Rahway, N. J., was subjected to analysis.

Hydrolysis of Proteins—Lyophilized proteins (2.0 to 3.9 mg) were hydrolyzed in 1.0 ml of constant boiling HCl for 24 hours at 110°C in an evacuated desiccator (25). Hydrolysates were evaporated to dryness over NaOH pellets in vacuo, dissolved in 0.2 N sodium citrate, pH 2.2, at concentrations equivalent to 2.4 to 4.0 mg per ml of protein, frozen, and stored at -10°C. Control studies carried out on arginine and each of the methylarginine standards showed that treatment under conditions of acid hydrolysis results in the formation of a minute quantity of ornithine, less than 1% of the arginine or methylarginine so treated.

Incubation of Proteins in Alkaline Urea Solution—Lysozyme (Worthington, LX-8F) and guinea pig myelin basic protein purified by Procedure e were dissolved at concentrations of 1.0 mg per ml in 0.01 M sodium glycinate, pH 10.6, made 2 M in deionized urea (20). The apparent pH of the solution before and after addition of protein was 10.9. Samples were removed during the course of the incubation, dialyzed exhaustively against 0.1 M acetic acid, and lyophilized. All procedures were carried out at 5°C. The proteins were hydrolyzed simultaneously in the same desiccator, and samples of hydrolysates equivalent to 0.8 mg were used.

1 The rat myelin basic protein used in this study consisted of a mixture of S and L proteins (21, 22) present in a ratio of approximately 3:1 (w/w).

ng of protein were analyzed back-to-back on the 30-cm Dur- 
rum DC-2A column.

RESULTS

Chromatography of Calibration Mixtures—Data typical of 
the results of over a dozen separations are presented in Table I. 
Peak elution times determined on consecutive runs agreed to 
within 1 min. When the pH of the starting buffer was 5.755 to 
5.805 (Chromatograms 1 and 2) a peak time difference of ap-
proximately 20 min was observed for the following pairs of 
consecutively emerging basic amino acids: lysine, N'-mono-

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chromatogram 1 (pH 5.800)</th>
<th>Chromatogram 2 (pH 5.800)</th>
<th>Chromatogram 3 (pH 5.835)</th>
<th>Chromatogram 4 (pH 5.835)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>60</td>
<td>61</td>
<td>58</td>
<td>59</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>64</td>
<td>66</td>
<td>63</td>
<td>64</td>
</tr>
<tr>
<td>5-Hydroxylysine</td>
<td>137</td>
<td>139</td>
<td>135</td>
<td>107, 111b</td>
</tr>
<tr>
<td>Ornithine</td>
<td>145</td>
<td>146</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>164</td>
<td>165</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>N'-Monomethyllysine</td>
<td>175</td>
<td>177</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>198</td>
<td>197</td>
<td>187</td>
<td>188</td>
</tr>
<tr>
<td>N'-Methylhistidine</td>
<td>217</td>
<td>215</td>
<td>203</td>
<td>220</td>
</tr>
<tr>
<td>N'-Methylhistidamine</td>
<td>225</td>
<td>228</td>
<td>222</td>
<td>222</td>
</tr>
<tr>
<td>Ammonia</td>
<td>289</td>
<td>274</td>
<td>266</td>
<td></td>
</tr>
<tr>
<td>Methylamine</td>
<td>306</td>
<td>356</td>
<td>345</td>
<td></td>
</tr>
<tr>
<td>N0, N'-Dimethylarginine</td>
<td>370</td>
<td>377</td>
<td>365</td>
<td></td>
</tr>
<tr>
<td>N0, N'-Dimethylarginine</td>
<td>421</td>
<td>430</td>
<td>416</td>
<td></td>
</tr>
<tr>
<td>N'-Monomethylarginine</td>
<td>438</td>
<td>440</td>
<td>434</td>
<td>436</td>
</tr>
</tbody>
</table>

* Elution times are in minutes. Chromatography was carried 
  out on a 30-cm column at a flow rate of 45 ml per hour. The initial 
  temperature of 28°C was raised to 55.5°C when the buffer was changed 
  at 200 min.

b Presumably hydroxylysine and allhydroxylysine. Which 
  time corresponds to which isomer was not determined.

The pH of the starting buffer was 5.80. N0, N'-MeArg and 
N0-MeArg: N0, N'-dimethyl- and N0-monomethylarginine, re- 
spectively. N0, N'-Dimethylarginine, if present, would have 
been eluted 20 min before N0, N'-dimethylarginine.
or included, in addition, exposure to alkaline pH (Procedures b and d). It was also true for the hydrolyzate of the bovine A1 protein, which had previously been reported to contain this dimethylarginine isomer (11). Interestingly, only a small amount of $N^\alpha,N^\alpha$-dimethylarginine and no $N^\alpha$-monomethylarginine were found in the hydrolyzate of the frog protein, and no methylarginine was detected in that of the carp. In the analyses of myelin basic proteins in which both $N^\alpha,N^\alpha$-dimethyl- and $N^\beta$-monomethylarginine were found the $N^\alpha$-monomethylarginine to $N^\alpha,N^\alpha$-dimethylarginine ratios ranged from a high of 7.4 in the rat to a low of 0.5 in the rabbit. In all of the myelin basic protein hydrolyzates the quantity of ornithine recovered could be adequately accounted for by the breakdown of arginine during acid hydrolysis.

As shown in Table II analysis of protein fractions removed from the turtle myelin basic protein during its chromatographic purification revealed relatively large amounts of $N^\alpha,N^\alpha$-dimethylarginine. It would appear highly probable, therefore, that the trace of $N^\alpha,N^\alpha$-dimethylarginine found in the turtle myelin basic protein, equivalent to 1 residue in approximately 1 out of every 200 basic protein molecules, results from a slight contamination and is not an integral part of the myelin basic protein molecule. It is of interest that the two protein fractions which contained relatively high amounts of $N^\alpha,N^\alpha$-dimethylarginine also contained unusually large amounts of ornithine.

Certain of the data in Table II can be expressed as moles per mole of protein. Since the studies of Baldwin and Carnegie (9) and of Brostoff and Eylar (10) provide evidence that only a single, specific arginyl residue of the myelin basic protein, arginine "107", is substituted, these values (moles per mole of protein) are equivalent to the fraction of myelin basic protein molecules which are methylated. As shown in Table III, between 0.033 and 0.35 of the molecules, depending upon the species source, contained disubstituted arginine, while mono-substituted arginine was present in 0.17 to 0.32 of the molecules. In none of the species examined did the extent of the methylation of a single arginyl residue even approach 100%.

Effect of Incubation in Alkaline Urea Solution on Lysine, Arginine, $N^\alpha,N^\alpha$-Dimethyl-, and $N^\beta$-Monomethylarginine in Myelin Basic Protein—Guinea pig protein which had never been exposed to alkaline pH or urea (purified by Procedure c) was incubated at 5°C in 2 M urea-0.01 M sodium glycinate, apparent pH 10.9.

### Table II

<table>
<thead>
<tr>
<th>Protein (and method of preparation)</th>
<th>$N^\alpha,N^\alpha$-Dimethylarginine</th>
<th>$N^\alpha,N^\alpha$-Monomethylarginine</th>
<th>Ornithine</th>
<th>% of total arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig (a)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.79</td>
<td>0.40</td>
</tr>
<tr>
<td>Guinea pig (c)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Bovine (c)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.10</td>
</tr>
<tr>
<td>Bovine (b)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.06</td>
<td>0.40</td>
</tr>
<tr>
<td>Bovine &quot;A1&quot;</td>
<td>0.00</td>
<td>0.00</td>
<td>0.96</td>
<td>0.11</td>
</tr>
<tr>
<td>Rat (c)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.34</td>
<td>0.07</td>
</tr>
<tr>
<td>Frog (c)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.21</td>
</tr>
<tr>
<td>Carp (c)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.30</td>
</tr>
<tr>
<td>Rabbit (b)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.73</td>
<td>0.06</td>
</tr>
<tr>
<td>Monkey (b)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.03</td>
<td>0.23</td>
</tr>
<tr>
<td>Human (b)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Turtle (d)</td>
<td>0.00</td>
<td>0.00</td>
<td>1.73</td>
<td>0.44</td>
</tr>
</tbody>
</table>

- Each set of values represents the result of a single analysis.
- Total arginine = arginine + methylarginines + ornithine.
- Samples analyzed contained 0.88 to 0.98 pmole of arginine.
- These abnormally low values and the presence of what appeared to be $N^\alpha$-monomethyllysine (0.18% of the lysine) suggest that this preparation may have been contaminated by other proteins, possibly histones.

### Table III

<table>
<thead>
<tr>
<th>Myelin basic proteins (and method of preparation)</th>
<th>Total arginine</th>
<th>$N^\alpha,N^\alpha$-Dimethylarginine</th>
<th>$N^\beta$-Monomethylarginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (c)</td>
<td>18</td>
<td>0.033</td>
<td>0.24</td>
</tr>
<tr>
<td>Guinea pig (a)</td>
<td>18</td>
<td>0.085</td>
<td>0.32</td>
</tr>
<tr>
<td>Bovine (b)</td>
<td>18</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Bovine &quot;A1&quot;</td>
<td>18</td>
<td>0.067</td>
<td>0.17</td>
</tr>
<tr>
<td>Turtle (d)</td>
<td>18</td>
<td>0.24</td>
<td>0.31</td>
</tr>
<tr>
<td>Monkey (b)</td>
<td>10</td>
<td>0.30</td>
<td>0.25</td>
</tr>
<tr>
<td>Human (b)</td>
<td>10</td>
<td>0.33</td>
<td>0.20</td>
</tr>
<tr>
<td>Rabbit (b)</td>
<td>18</td>
<td>0.35</td>
<td>0.19</td>
</tr>
</tbody>
</table>

- Assumed number, consistent with data obtained from total amino acid analyses.
- Total arginine = arginine + methylarginines + ornithine.
- As percent of ornithine + arginine in the protein.
- As percent of ornithine + arginine + methylarginines in the protein.
- Theoretical value, 6.00 (28).

### Table IV

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Guinea pig myelin basic protein</th>
<th>Lysozyme</th>
<th>Guinea pig myelin basic protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine/ornithine</td>
<td>Ornithine¹</td>
<td>Lysine/ornithine¹</td>
<td>Ornithine¹</td>
</tr>
<tr>
<td>0</td>
<td>5.77</td>
<td>0.079</td>
<td>1.54</td>
</tr>
<tr>
<td>30</td>
<td>5.60</td>
<td>0.122</td>
<td>1.52</td>
</tr>
<tr>
<td>120</td>
<td>5.55</td>
<td>0.140</td>
<td>1.46</td>
</tr>
</tbody>
</table>

- As percent of ornithine + arginine in the protein.
- As percent of ornithine + arginine + methylarginines in the protein.
- Theoretical value, 6.00 (28).

- During high pH chromatography the myelin basic proteins are exposed to this solvent for approximately 18 hours; for the large scale preparation the exposure is approximately 1 hour.
a small decrease in the lysine to histidine ratio, equivalent to only a 4 to 5% loss of lysine after 120 hours. Some slow carbamylation of lysine with conversion to homocitrulline would be expected under these conditions (29).

The stability of arginine and the two methylarginines under these conditions was clearly evident. The increase in ornithine resulting from incubation for 120 hours represented a possible maximum loss of arginine (lysosome) or arginine plus methylarginines (myelin basic protein) of less than 0.1%, while the $N^\gamma,N^\delta$-dimethylarginine-histidine ratios showed that no significant loss of $N^\gamma,N^\delta$-dimethylarginine occurred. Although $N^\gamma$-monomethylarginine and arginine were poorly resolved in this series of analyses, manual integration of the methylarginine shoulder on the arginine peak of each of the three samples and calculation of the approximate $N^\gamma$-monomethylarginine to histidine ratios indicated no significant changes. Since $N^\gamma,N^\delta$-dimethylarginine was absent from the myelin basic protein examined, the stability of this amino acid at pH 10.9, 5%, was not directly tested. It seems reasonable to assume that it is not significantly more labile than $N^\gamma,N^\delta$-dimethyl- and $N^\gamma$-monomethylarginine. Reporter and Corbin (18) reported that $N^\gamma,N^\delta$-dimethylarginine is not affected by incubation at pH 9.5, 45°, for 45 min. In the present studies this amino acid was found in turtle proteins which had been subjected to high pH chromatography.

DISCUSSION

As far as we are aware the present study is the first to describe the direct determination of arginine, $N^\gamma$-monomethylarginine, and the two structural isomers of dimethylarginine in total acid hydrolyzates of the proteins. The results obtained in some cases confirm and in other cases contradict previous reports regarding the methylarginine contents of myelin basic proteins.

The $N^\gamma,N^\delta$-dimethylarginine values for our turtle, bovine, and rabbit proteins are essentially the same as the dimethylarginine values reported for these species by Brostoff and Eylar (10), but our values of $N^\gamma,N^\delta$-dimethylarginine for the remaining species are considerably lower than the dimethylarginine values they reported. It would appear from our data that the bovine protein does not contain up to 0.8 mole of $N^\gamma$-monomethylarginine per mole of protein as previously claimed (10, 30). Our value for the fraction of the human protein containing $N^\gamma,N^\delta$-dimethylarginine at position 107 is approximately one-half the value reported by Baldwin and Carnegie (9) and approximately one-half the value of dimethylarginine reported by Brostoff and Eylar (10). It is of interest, however, that our value of 1.6 for the $N^\gamma,N^\delta$-dimethylarginine to $N^\gamma$-monomethylarginine ratio is in excellent agreement with the value of 1.7 calculated from the data of Baldwin and Carnegie (9). Our value of $N^\gamma,N^\delta$-dimethylarginine in the rat protein is only one-tenth that reported for dimethylarginine in this species by Brostoff and Eylar (10). Whether the discrepancies in $N^\gamma,N^\delta$-dimethyl- and $N^\gamma$-monomethylarginine contents are the result of differences in the methods of analysis or reflect actual variations in the methylation of the protein remains to be determined.

$N^\gamma,N^\delta$-Dimethylarginine was completely absent in hydrolyzates of myelin basic proteins prepared from eight out of nine animal species; the trace present in that of the turtle protein was in all probability a contaminant. Its conspicuous absence, even in the bovine A1 protein itself, cannot be reconciled with earlier claims (10, 11, 30) that an appreciable fraction of the dimethylarginine present in the bovine myelin basic protein occurs as the unsymmetrical isomer.

Acknowledgments—We wish to thank Dr. M. Report for samples of methylarginines, Dr. S. Levine for frog and carp spinal cords, and Dr. F. H. Eylar for a sample of his A1 protein. We also wish to thank Dr. R. S. Adelstein for valuable advice and Dr. M. W. Kies for her constant support and encouragement.

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