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Note

Analysis of methylated amino acids by high-performance liquid chromatography: methylation of myelin basic protein

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The identification and quantitative analysis of methylated amino acids has been achieved by a variety of techniques including paper electrophoresis [1], chemical degradation [1–4], high-performance liquid chromatography (HPLC) [5,6], and conventional amino acid analysis [7,8]. When the amino acid analyzer is used, basic amino acids must be separated from the rest of the protein hydrolysate by ion-exchange chromatography prior to injection. The elution times reported are of the order of 7 h with sensitivities generally limited to 1 μmol [2,7]. Furthermore, arginine and lysine and their monomethyl derivatives are not well separated with this system.

Reversed-phase HPLC has emerged as a fast and efficient method for the analysis of amino acids and extensive literature is available on various pre- and post-column derivatization techniques [9]. Among these methods, the use of phenylthiocarbamyl derivatives of protein hydrolysates seems to overcome most of the problems encountered with other derivatization techniques and samples as small as 1 pmol can be conveniently analyzed [10–12]. We report in this communication that this technique allows a facile and rapid separation and quantification of monomethylarginine, N^G,N^G- and N^G,N'^G-dimethylarginine, 3-methylhistidine and mono- and trimethyllysine from other naturally occurring amino acids. This technique is used to probe the extent and nature of the methylation of bovine myelin basic protein (MBP) and histone fraction F3.

EXPERIMENTAL

Materials

Phenylisothiocyanate (PITC), a standard mixture of amino acids (2.5 μmol/ml in 0.1 *M* hydrochloric acid) and constant-boiling hydrochloric acid (sequanal

0378-4347/87/$03.50

grade) were purchased from Pierce (Rockford, IL, U.S.A.). Histones, N^G,N'^G-dimethylarginine, 3-methylhistidine, monomethyllysine, and ornithine were from Sigma (St. Louis, MO, U.S.A.). N^G,N^G-Dimethylarginine was from Calbiochem (San Diego, CA, U.S.A.), and trimethyllysine was purchased from Bachem (Terrance, CA, U.S.A.). Monomethylarginine was synthesized by the method described by Corbin and Reporter [13]. Sodium acetate, triethylamine, and acetonitrile (HPLC grade) were obtained from Aldrich (Milwaukee, WI, U.S.A.). S-Adenosyl-L-[methyl-^{3}H]-methionine([^{3}H]AdoMet) was purchased from Amersham (Arlington Heights, IL, U.S.A.); glass-distilled water was used throughout.

Sample preparation

Myelin basic protein (MBP) was prepared from bovine brain white matter as previously described [14] and homogeneity confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis; protein concentrations were measured by the Bradford method [15]. The enzyme S-adenosyl-L-methionine (AdoMet):MBP methyltransferase was isolated from bovine brain as described elsewhere [16]. [^{3}H]AdoMet was diluted to 110 dpm/pmol. The methylation of MBP by AdoMet was assayed [16] by adsorbing MBP from the assay mixture onto nitrocellulose filters coated with carboxymethyl (CM) cellulose (Schleicher & Schuell, Keene, NH, U.S.A.). The standard assay contained MBP (1.0 mg), methyltransferase enzyme preparation, [^{3}H]AdoMet (typically 110 dpm/pmol), and sufficient 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (10 m*M*, pH 7.2) to give an assay volume of 0.5 ml. Assays were performed in 1.5-ml polyethylene microcentrifuge tubes suspended in a thermostated water bath at 37°C. The reaction was terminated by the addition of 1.0 ml of ice cold MOPS buffer followed by filtration of the mixture through the CM filter on a vacuum manifold. The filter was washed with 400 ml of buffer to remove retained [^{3}H]AdoMet and then transferred to a scintillation vial. The protein was eluted from the membrane by the addition of 1.0 ml of 1 *M* hydrochloric acid. For amino acid analysis, protein samples were dialyzed against 0.1 *M* acetic acid and lyophilized prior to hydrolysis. Stock solutions were 0.01 *M* for single amino acids and 1 mg/ml for the protein solutions. Solutions containing 30 μg of protein were dried in vacuum and hydrolyzed at 110°C for 22 h using vapor phase hydrolysis with 6 *M* constant-boiling hydrochloric acid. Protein hydrolysates, 10 μl of the standard mixture of amino acids (250 nmol each), and 10 μl of the methylated amino acid solution (100 nmol) were derivatized with phenylisothiocyanate to their corresponding phenylthiocarbamyl products. Hydrolysis and derivatization were performed on a Waters Pico-Tag workstation [10].

Reversed-phase separation of methylated amino acids

Analyses were carried out with a Hitachi HPLC system consisting of a gradient processor (Model 655 A61-1) and a ternary pump with a variable-wavelength detector set at 254 nm. An Ultrasphere ODS C_{18} reversed-phase column (Beckman Instruments, San Ramon, CA, U.S.A.) was used (250×4.6 mm; 5 μm particle diameter) coupled to a guard column packed with C_{18} pellicular media (Whatman, Clifton, NJ, U.S.A.). The temperature was maintained at 35±1°C

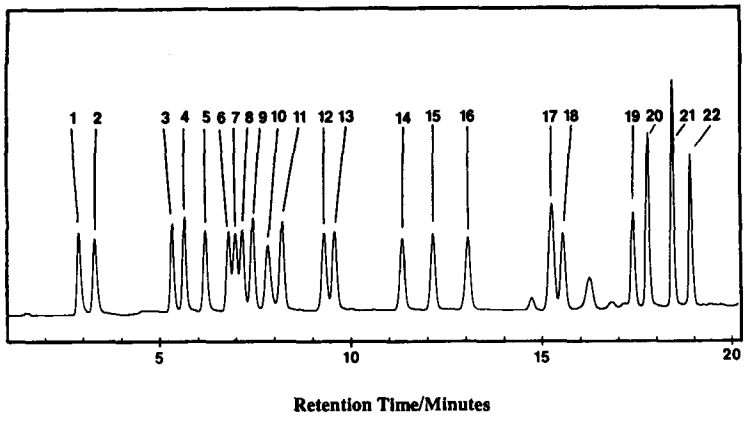

Fig. 1. Separation of phenylthiocarbamyl amino acid standards by reversed-phase HPLC. Samples contained 500–600 pmol of each amino acid. Peaks by order of elution: 1 = Asp; 2 = Glu; 3 = Ser; 4 = Gly; 5 = His; 6 = Thr; 7 = Arg; 8 = Ala; 9 = Pro; 10 = 3-methylhistidine + NH_3; 11 = monomethylarginine; 12 = N^G,N^G-dimethylarginine; 13 = N^G,N'^G-dimethylarginine; 14 = Tyr; 15 = Val; 16 = Met; 17 = Ile + Cys; 18 = Leu; 19 = Phe; 20 = Orn; 21 = Lys; 22 = N^ε-monomethyllysine. Trimethyllysine has a retention time of 7.8 min under these conditions and elutes midway between proline and 3-methylhistidine, peaks 9 and 10.

using a column jacket linked to a circulating water bath. Elution solvent A consisted of 0.14 *M* sodium acetate mixed with 970 μl/l triethylamine, titrated to pH 6.4 with glacial acetic acid, filtered through a 0.45-μm nylon 66 filter, and combined with 6% (v/v) of acetonitrile. Solvent B was acetonitrile–water (60:40). The flow-rate was kept constant at 1.5 ml/min. The elution was carried out using the following gradient system: 0 min, 100% A–0% B; 1.0 through 3.0 min (isocratic), 90% A–10% B; 13.0 min, 70% A–30% B; 16.0 min, 45% A–55% B; 20.0 min, 0% A–100% B. At the end of each analysis the column was washed with 100% solvent B for 15 min and pre-equilibrated with solvent A for an additional 5 min prior to the next analysis.

RESULTS AND DISCUSSION

The separated phenylthiocarbamyl amino acids were identified by their retention times as shown in Fig. 1. The analysis required 19 min followed by a 15-min wash during which time the PITC by-products eluted. In general, methylated amino acids can be analyzed directly from a protein hydrolysate without the prior separation of basic amino acids. This extra step, however, may be required for the accurate quantification of trimethyllysine in the presence of proline (peak 9, Fig. 1). We have found, however, that overlap such as this can often be eliminated by slightly increasing or decreasing the column temperature by about 2°C. The system was tested for reproducibility by injecting samples on different days and for several consecutive analyses. In general, the retention times did not vary more than 5 s, and the area variation was less than 5%. The temperature chosen for the analysis (35°C) represents an optimization of conditions for the separation of the derivatized methylated amino acids. The sensitivity of both absolute and

TABLE I

AMINO ACID COMPOSITION OF BOVINE MYELIN BASIC PROTEIN (MBP) AND CALF THYMUS HISTONE F3

Retention time (min)	Residue	MBP (mol per 100 mol)		Histone F3 (mol per 100 mol)	
		Observed	Calculated	Observed	Calculated
2.89	Asp	8.0	6.5	5.6	4.4-5.2
3.29	Glu	6.7	5.9	9.9	10.2-11.7
5.32	Ser	10.5	11.2	3.7	3.8-5.1
5.63	Gly	14.2	14.7	5.6	5.8-6.5
6.18	His	5.5	5.9	2.3	2.0-2.4
6.79	Thr	4.6	4.1	6.0	6.4-6.9
6.97	Arg	9.9	10.6	12.9	10.8-13.6
7.14	Ala	8.2	8.2	12.7	12.6-13.5
7.42	Pro	7.5	7.1	4.8	4.4-5.1
8.17	N^G-Arg	0.36(0.61)*	ca. 0.24-0.47**	0.0	0.0
9.57	N^G,N'^G-Arg	0.18(0.31)*	ca. 0.11**	0.0	0.0
11.35	Tyr	1.7	2.4	2.7	2.1-2.5
12.16	Val	1.8	1.8	5.4	4.6-6.4
13.08	Met	1.4	1.2	1.2	1.5
15.29	Ile	1.8	1.8	4.2	4.8
15.59	Leu	5.8	5.9	10.6	9.0
17.42	Phe	3.9	4.7	1.8	2.0-2.6
17.83	Orn	0.094	0.0	0.078	0.0
18.48	Lys	7.2	7.6	10.1	9.8-10.1
18.96	N^ϵ-Lys	0.0	0.0	0.4	0.5-0.6

*Calculation based on 170 amino acid residues per mol of MBP.
**Data from Eylar and Brostoff [3].

relative retention times to temperature points out the importance of accurate temperature control in obtaining reproducible results.

The amino acid hydrolysates of (MBP) and histone fraction F3 were analyzed under the same conditions. The amino acid peak areas were normalized against external standards and the molar ratios calculated as percentages of the total mol or amino acids recovered. The observed amino acid compositions are compared with literature values in Table I. The methylation of MBP is known to occur on a single arginine residue (Arg-108) although the molar ratio of the methylated residue and the isomer distribution is still controversial [3,7,8]. We have found a total of 0.92 mol of total methylarginine per mol of protein with an isomer distribution of 0.61 mol per mol of protein for monomethylarginine and 0.31 mol per mol of protein for N^G,N'^G-dimethylarginine; no traces of the unsymmetrical isomer were found. Values of 0.18–0.8 mol/mol for monomethylarginine and 0.12–0.20 mol/mol for N^G,N'^G-dimethylarginine are reported in the literature [3,7,8], giving values between 0.3 and 1.0 for total mol of methylated residue per mol of protein. Unsymmetrical dimethylarginine has been reported to be a minor component of MBP by Eylar et al. [17], but this report has not been confirmed

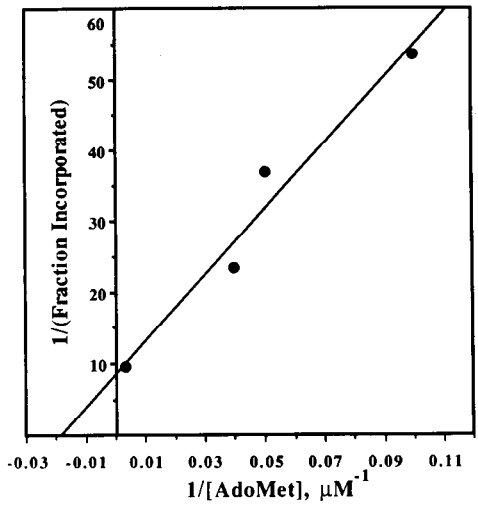

Fig. 2. Double reciprocal plot showing the dependence of the limiting methyl group incorporation into bovine myelin basic protein (MBP) from S-adenosyl-L-methionine (AdoMet), as catalyzed by the enzyme AdoMet:MBP methyltransferase, on the concentration of AdoMet. Assay mixtures contained 1.0 mg of MBP and methyltransferase enzyme solution in 0.5 ml 3-(N-morpholino)propanesulfonic acid buffer, pH 7.2, 37°C; limiting incorporation was observed at approximately 20 min.

by other workers [4]. The discrepancies in the data may be due to differences in the sample preparation, in measurement techniques, or may represent actual differences in the in vivo methylation of MBP due to the possible persistence of a demethylase activity [16].

In order to further corroborate the ratios of methylated Arg-108 in the MBP samples used in this work, we have examined the incorporation of methyl groups from [^{3}H]AdoMet into the protein as catalyzed by the enzyme AdoMet:MBP methyltransferase from bovine brain. As previously reported [16], using a fixed concentration of enzyme, MBP and AdoMet in the assay, a limiting number of methyl groups can be incorporated into the protein during the standard 20–30 min incubation period. This limiting value increases with increasing AdoMet concentration and begins to saturate with [AdoMet] approximately 300 μM. The limiting values observed at several AdoMet concentrations are plotted in the double reciprocal plot in Fig. 2 and, although there is some scatter giving rise to uncertainty in the slope, the intercept value (limiting methyl incorporation) is well defined by the data. This limiting value corresponds to a mol fraction of about 0.11 ± 0.02 which agrees very well with our determination of ca. 8% unmethylated Arg-108 in these basic protein samples. These data further suggest that the enzyme used in these previous studies does not methylate monomethylarginine (present as 61% of the Arg-108 in the sample) and suggests that the mixture of mono-, di- and unmethylated MBP which is observed in vivo arises from stepwise methylation of the protein, perhaps by separate enzymes.

The amino acid composition of histone F3 fraction (arginine-rich) correlates quite well with the literature data. The variation in the reported composition is

caused by the association of the protein with other proteins of similar solubility, and by formation of polymers through interacting disulfide bridges [18,19]. Consistent with literature data, monomethyllysine accounts for 3.8% of the total lysine and no other methylated species are observed.

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