IDENTIFICATION OF ε-N-MONOMETHYLLYSINE AND ε-N-TRIMETHYLLYSINE IN RABBIT SKELETAL MYOSIN

W. Michael Kuehl and Robert S. Adelstein
Section on Cellular Physiology, Laboratory of Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Md. 20014.

Received August 4, 1969

SUMMARY. Adult rabbit skeletal myosin has been shown to contain both ε-N-monomethyllysine and ε-N-trimethyllysine but no ε-N-dimethyllysine. These two unusual amino acids occur in an approximately 1:2:1 ratio with 3-methylhistidine, a known component of myosin. The same methylated amino acids are also present in myosin derivatives, namely heavy meromyosin and subfragment I, in similar molar ratios as in myosin, except for ε-N-monomethyllysine which is substantially reduced in subfragment I.

Methylated lysine derivatives have been reported to occur in several proteins. Specifically, ε-N-monomethyllysine (MML)[1] has been found in some species of Salmonella flagella (1) and histones from various sources (2). ε-N-dimethyllysine (DML) has been found to be present in certain histones (3). More recently ε-N-trimethyllysine (TML) has been shown to occur in histones (4) and in two species of cytochrome c (5). Having noted assymmetry of the trailing edge of the lysine peak from a myosin hydrolysate run on a 20 cm Beckman analyzer column with the standard pH 5.28 sodium citrate buffer, we were prompted to investigate myosin for the possible presence of methylated lysine derivatives. These studies resulted in identification of both MML and TML in rabbit skeletal myosin.

MATERIALS AND METHODS

Myosin was prepared as outlined previously by Kielley and Harrington (6) except that final purification was achieved by passing the material

1. Abbreviations used: ε-N-monomethyllysine (MML); ε-N-dimethyllysine (DML); ε-N-trimethyllysine (TML); 3-methylhistidine (3MH); heavy meromyosin (HMM); light meromyosin (LMM); subfragment I(HMM-S1).

through a column of hydroxyapatite (personal communication, W.W. Kielley). Heavy meromyosin (HMM) (trypsin) and light meromyosin (LMM) (cyanogen bromide) were prepared as described by Young et al. (7,8). Subfragment I (HMM-S1) was a gift of Dr. Susan Lowey and was prepared using insoluble papain as outlined by Lowey et al. (9). The low molecular weight component(s) of myosin were removed by precipitation of myosin from 2M urea at low ionic strength as described by Gazith et al. (10); approximately 12% of the protein from the original myosin preparation was removed by two cycles of this procedure. Tropomyosin was prepared by the method of Hartshorne and Mueller (11). Actin was prepared by the method of Adelstein et al. (12) as modified by Rees and Young (13). The myosin, HMM, LMM, and tropomyosin were gifts of Dr. W.W. Kielley.

Samples for amino acid analysis were taken up in 6N HCl, sealed under vacuum after thrice flushing with nitrogen, and hydrolyzed for 72 hours at 110°C., since hydrolysis of the proteins investigated was found to be incomplete after 24 hours. All the basic amino acids listed in Table I were recovered quantitatively from standard mixtures that were hydrolyzed for 72 hours. TML was assumed to have the same ninhydrin color value as arginine (5).

MML, DML, and 3MH were purchased commercially. TML was synthesized. (5)

Descending paper chromatography: Solvent I. Phenol-cresol-borate as described by Stewart (14) and modified by Delange et al. (5); Solvent II. 2-propanol-ammonia-water (8:1:1) as described by Perry et al. (15). All chromatograms were run on Whatman 3MM paper for 12-16 hours.

Paper electrophoresis: The buffer used was 0.04M NH_4HCO_3-1mM EDTA adjusted to pH 10.04 with ammonia. Electrophoresis was performed at 3000 volts for 50 minutes using Whatman 3MM paper 65 cm. in length.

RESULTS AND DISCUSSION

Table I provides a summary of the approximate times of elution of

TABLE I

Retention Times of Basic Amino Acids with Various Amino Acid Analyzer Systems

Residue	System A pH5.28, 50°C	System B pH5.84, 27°C	System C pH5.28, 27°C
Ornithine	175	154	190
Lysine	182	163	204
MML	199	189	231
DML	199	203	244
TML	190	211	244
Histidine	231	225	323
Ammonia	284	245	297
3MH	246	257	372

In each case a 55 cm column was used and elution at a flow rate of 68ml/hour was carried out with 0.35N sodium citrate buffer at the pH and temperature indicated. Pattern A was obtained on a Beckman 120 amino acid analyzer, using AA15 Beckman resin. Patterns B and C were obtained on another Beckman analyzer, using UR30 Beckman resin.

basic amino acids from an amino acid analyzer using three different systems for elution. System A (2) resolves all components except DML which co-elutes with MML. Although system B(3) resolves all of the basic residues, the separation of 3MH from ammonia was usually inadequate because of the relatively large amounts of the latter compared to the former. System C, as modified from systems A and B, adequately resolves all of the amino acids listed except DML and TML, which emerge together.

Acid hydrolysates of myosin were quantitatively analyzed with each of the three systems described in Table I. In each case, peaks eluting in the positions expected for ornithine[2], lysine, MML, TML, histidine, ammonia, and 3MH were observed. The A_{440}/A_{570} ratios of each component resolved from myosin agreed with those expected for the amino acids listed. Chromatographic analysis with system B revealed that despite the apparent presence of both MML and TML in myosin, DML was not

2. Although variable amounts of ornithine were consistently present in quantities greater than 0.3moles/100,000 grams of myosin hydrolysate, the significance of this finding is uncertain since this amino acid is frequently present as a contaminant.

present. Addition of DML to the myosin hydrolysate confirmed that this amino acid is resolved from the presumptive peaks of MML and TML with system B.

In order to isolate sufficient quantities of the unknown peaks for further characterization in paper chromatographic systems, fractions were collected from the analyzer using system C. 55mg of myosin hydrolysate was applied to the analyzer, and all of the effluent between the lysine and ammonia peaks was collected directly from the solumn. After being desalted essentially as outlined by Dreze et al. (16), 10% of the isolated material was run on the analyzer using system A. This analysis showed 0.048μmol of a peak with a retention time identical to TML and 0.018μmol of a peak with the same retention time as MML (or DML). Only very small amounts of ornithine ($<$.007μmol), lysine ($<$.006μmol) and histidine ($<$ .003μmol) were present. The

TABLE II

Rf Values of Standard Amino Acids and Unknowns from Myosin

Solvent I (Phenol:Cresol:Borate)		Solvent II (2 propanol:NH_4OH:H_2O)	
Amino Acid Standards	Rf	Amino Acid Standards	Rf
Ornithine	0.12	TML	0.10
Lysine	0.20	Arginine	0.14
Arginine	0.40	Ornithine	0.15
Histidine	0.46	Lysine	0.18
MML	0.53	Histidine	0.22
DML	0.82	MML	0.26
TML	0.93	DML	0.45
Myosin Unknowns		Myosin Unknowns	
I-1	0.49	II-1	0.10
I-2	0.88	II-a	0.14
		II-b	0.18
		II-2	0.28

The desalted material isolated from a myosin hydrolysate (see text) was examined in two descending paper chromatographic systems. Solvent I was applied directly to the sample. In contrast, application of Solvent II was performed after a first dimension of electrophoresis in 1mM EDTA - 0.04M NH_4HCO_3 (pH 10.04).

amounts of TML and MML indicated that recovery from the starting material was quantitative (see Table III). Descending paper chromatograms were run using two different solvent systems (Table II). The Rfs of known amino acids are given in the upper portion of this table and the Rfs of the ninhydrin positive unknowns are given below. With solvent I only two major ninhydrin positive spots were seen although there was a ninhydrin smear at the origin. The Rfs of the two ninhydrin spots were consistent with the Rfs for MML and TML in this system, if allowances are made for retardation of these spots because of small amounts of salt, which despite the desalting procedure, were still present.

Because of a similar salt effect with solvent II the unknown peaks were electrophoresed on paper at pH 10.04 prior to chromatography with this solvent (i.e. a peptide map). In this electrophoretic system the only amino acids migrating toward the cathode are lysine, MML, DML, TML, arginine, and ornithine, which are partially separated. The Rfs given in the right half of Table II are for the myosin unknowns run in solvent II after electrophoresis, and apply only to the chromatography dimension. Two major ninhydrin spots were apparent, one with an Rf of TML(II-I) and one with an Rf very close to MML(II-2). Two light ninhydrin spots (II-a and II-b) with Rfs virtually identical to lysine and ornithine or arginine were also seen.

On the basis of results utilizing three different analyzer systems and two different paper chromatography solvents, it is concluded that the myosin unknowns are MML and TML.[3]

Table III lists the amounts - as determined with the amino acid analysis systems described above - of MML, TML, and 3MH in adult rabbit

3. A recent report by Hardy and Perry (18) in which they studied the incorporation of a ^{14}C-labelled methyl group from S-adenosyl methionine into myofibrillar protein *in vitro*, describes analytical separation of three ^{14}C fractions co-eluting with 1)MML and/or DML; 2)3MH, and 3) an unknown.

TABLE III

Content of ε-N-monomethyllysine, ε-N-trimethyllysine, and 3-methylhistidine in Adult Rabbit Skeletal Myosin and its Proteolytic Fragments

Protein	Moles per 100,000 grams of Protein MML	TML	3MH	#3MH
Myosin	0.27	0.78	0.31	0.35
*"Purified" Myosin	0.29	0.81	0.26	---
Heavy Meromyosin	0.53	1.04	0.49	0.54
Subfragment I	0.29	1.50	0.68	0.78
Light Meromyosin	absent	absent	absent	absent

Methods of analysis are described in the text. #The figures for 3MH in the last column are calculated from the data presented in Table 3 from Johnson et al. (17). *"Purified" myosin refers to a preparation of myosin from whith the low molecular weight component(s) had been removed as described in the text.

skeletal myosin and its derivatives. For comparison, the data of Johnson et al. (17), which provides quantitation of the 3MH content in myosin and its derivatives, is included in this table. As may be seen, removal of the low molecular weight component(s) of myosin had no significant effect on the quantitation of any of the three methylated amino acids. Several conclusions are justified by this data: (1) all three methylated amino acids are present in the HMM portion of myosin but are not present in detectable amounts in LMM; (2) the TML:MML:3MH proportions are approximately 2-3:1:1 in both myosin and HMM; (3) the molar content of both TML and 3MH in HMM-S1, are comparable to their molar contents in the subunits of myosin and HMM; and (4) MML, which is substantially reduced relative to TML and 3MH in HMM-S1, is clearly not present in stoichiometric quantities in this fragment (i.e. only 0.4 moles per 130,000 grams). The presence of less than stoichiometric amounts of MML in HMM-S1 suggests that this unusual residue is located in a papain sensitive region. Although it appears that there are about 1 mole each of 3MH and MML, and 2 moles of TML per myosin (or HMM) subunit, exact

molar ratios cannot be calculated because of the uncertainty in homogeneity and subunit structure of myosin and its derivatives.

Neither actin nor tropomyosin B contained detectable amounts of any of the methylated lysine derivatives.

It is of particular interest that in view of our findings myosin presently provides a unique example of the occurrence of MML and TML without significant amounts of DML.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. W. W. Kielley for his valuable advice and encouragement, to Miss Mary Ann Conti for excellent technical assistance and to Mrs. Carol Dunham for the amino acid analyses.

REFERENCES

1. Ambler, R.P. and M.W. Rees, Nature, 184, 56 (1959).
2. Murray, K., Biochemistry, 3, 10 (1964).
3. Paik, W.K. and S. Kim, Biochem. Biophys. Res. Comm., 27, 479 (1967).
4. Hempel, K., H.W. Lange, and L. Birkofer, Die Naturwissenschaften, 55, 37 (1968).
5. Delange, R.J., A.N. Glazer, and E.L. Smith, J. Biol. Chem., 244, 1385 (1969).
6. Kielley, W.W. and W.F. Harrington, Biochim. Biophys. Acta, 41, 401 (1960).
7. Young, D.M., S. Himmelfarb, W.F. Harrington, J. Biol. Chem., 239, 2822 (1964).
8. Young, D.M., Proc. Natl. Acad. Sci. U.S., 58, 2393 (1967).
9. Lowey, S., H.S. Slayter, A.G. Weeds and H. Baker, J. Mol. Biol. 42, 1 (1969).
10. Gazith, J., S. Himmelfarb, and W.F. Harrington, in press, J. Biol. Chem.
11. Hartshorne, D. and H. Mueller, Biochim. Biophys. Acta, 175, 301 (1969).
12. Adelstein, R.S., J.E. Godfrey, and W.W. Kielley, Biochem. Biophys. Res. Comm., 12, 34,(1963).
13. Rees, M. and D.M. Young, J. Biol. Chem. 242, 4449,(1967).
14. Stewart, I., J. Chromatog., 10, 404, (1963).
15. Perry, T.L., S. Diamond, and S. Hansen, Nature, 222, 668, (1969).
16. Drèze, A., S. Moore, and E.J. Bigwood, Anal. Chim. Acta, 11, 554, (1954).
17. Johnson, P., C.I. Harris, and S.V. Perry, Biochem. J., 105, 361, (1967).
18. Hardy, M.F. and S.V. Perry, Nature, 223, 300, (1969).