N^G,N^G-DIMETHYLARGININE IN MYOSIN DURING MUSCLE DEVELOPMENT

Minocher Reporter and James L. Corbin

C. F. Kettering Research Laboratory
Yellow Springs, Ohio 45387

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SUMMARY: N^G,N^G-Dimethylarginine (unsym-DMA) has now been found to be present in myosin prepared from developing leg muscle. Neither monomethylarginine nor sym-DMA ($N^GN'^G$-dimethylarginine) could be detected in our preparations. Cultured muscle cell myosin contains up to four residues of this amino acid per 5×10^5 grams of protein. Myosins from leg muscle of chick embryos and neonatal rats contained less than two residues of unsym-DMA, while none was detected in adult chicken or rat myosins. Cardiac myosins from developing as well as mature animals lacked or contained very little unsym-DMA. Actin was devoid of this amino acid.

INTRODUCTION: Myosin prepared from adult vertebrate white muscle fibres contains both N^ϵ-mono- and trimethyl-lysines (MML and TML) as well as 3-methylhistidine (3-MeHis) (1,2). Myosin from adult cardiac muscle, red fibres of vertebrates, or fetal myosin contains little or no 3-MeHis (3,4). The methylated lysines are present in all vertebrate myosins, although the amounts may vary with different muscle types and age of animals sampled (4). On the other hand, actin from a wide variety of species consistently contains one 3-MeHis residue per mole (5,6). We now wish to report evidence that another methylated basic amino acid, N^G,N^G-dimethylarginine (unsym-DMA), is associated with differentiating striated muscle myosin but is not present in adult myosin.

MATERIALS AND METHODS: Myosin was prepared in all cases according to modifications presented by Kuehl and Adelstein (4). Myosin

preparations were monitored by electrophoresis on 5% acrylamide gels (7) to ensure minimal contamination from actin. The electrophoresis pattern showed a heavy main band of myosin and two satellite bands.

Actin was prepared by the method of Carstens and Mommaerts (8) and reprecipitated twice to yield preparations giving a single band in acrylamide gel electrophoresis and mobility values (7) of 0.44 and 0.36 with 5% and 10% gels runs for 4 and 4-1/2 hours respectively.

Rat muscle cells were cultured as described earlier (10).

Samples were hydrolyzed (in vacuo) at 110°C in 6N HCl for 24 hours (less than 10 mg protein) or 48 hours (10 to 30 mg protein), and were then dried in vacuo over sodium hydroxide. The ammonia content was reduced by a 45 minute incubation (45°C) at pH 9.5 followed by drying over sulfuric acid in vacuo. This treatment does not affect the guanidine group of arginine or of its methylated derivatives.

1- and 3-MeHis, MML, and dimethyllysine (DML) were obtained commercially (CalBioChem and Cyclo Corp.). TML was synthesized by alkylation of the copper complex of lysine. The methylated arginines were prepared by the general method of Kakimoto and Akazawa (9) except that thiourea derivatives were used instead of urea derivatives; this modification led to better yields.

A spherical sulfonated cation exchange resin was used for these analyses. This resin was 8% crosslinked and 12-15 microns in sizeand was packed in a 30 x 0.9 cm column (Mark Instrument Co., Villanova, Pa.). Routine analyses were made on a Model 8000B Phoenix Analyzer at 52.5°C with elutions performed at pH 4.26 using 0.35 N citrate buffer pumped at 60 ml per hour. The elution times for the basic amino acids in minutes are: TML 100, Lys 120, MML 126, ammonia 130, 1-MeHis 135, His 145, 3-MeHis 160, unsym-DMA 205,

sym-DMA 215, Arg 265. The presence of residual ammonia and an overload of lysine usually interfered with the resolution of MML. For optimal analysis 5 to 9 mg of myosin are required in this system.

RESULTS AND DISCUSSION: The methylated form of arginine was initially noted as a single radioactive product which migrates slower than arginine upon paper electrophoresis of muscle cell cytosol protein hydrolysates (10). These were prepared from cultures of muscle cells which had been labelled with methionine-methyl-^{14}C (Met). Since that time, preparations of myosin and cytosol or nascent polyribosomal proteins have been isolated from more than thirty different monolayer muscle cell cultures which had been treated with radioactive methionine or arginine. In every case, radioactivity corresponding to unsym-DMA could readily be detected after electrophoretic and chromatographic separations. Densitometric tracings of such autoradiograms show distinct peaks for the origin, neutral amino acids with Met, followed by 3-MeHis, unsym-DMA, MML and TML.

The identification of the unknown substance as unsym-DMA was established by a comparison of its properties with those of the synthetic amino acid using paper chromatography, electrophoresis, and behavior on the amino acid analyzer. Cells labelled with tritiated arginine, arginine-guanidino-^{14}C or methionine-methyl-^{14}C all yielded a myosin hydrolysate which contained a product identical to unsym-DMA when examined by electrophoresis in Offord's buffer (10), chromatography in Stewart's system (11), or a two-dimensional combination of these. An example of electrophoretic separation is shown in Figure 1. The substance was not observed in cells labelled with lysine-^{14}C. The unknown had distinctly different properties from those of sym-DMA or the dimethylornithines in column chromatography. After electrophoresis, and co-chromatography with the authentic com-

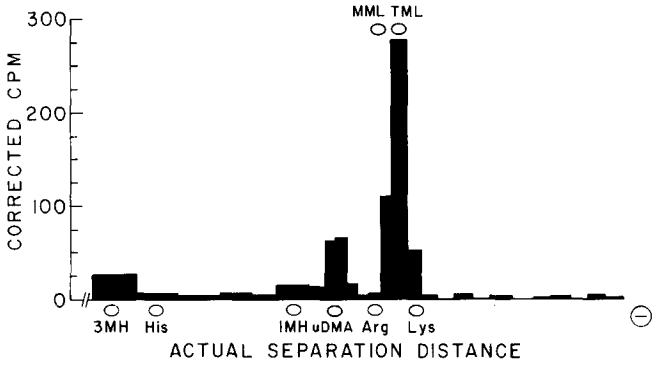

Figure 1. Radioactivity recovered after electrophoresis of myosin hydrolysate preparation from a 9 day old monolayer culture of rat leg muscle. Cells were incubated with methionine-methyl-^{14}C at a level of 0.2 µCi/ml medium for two hours. (S.A. of methionine originally was 53.6 mCi/mM). The myosin hydrolysate (see text for details) was subjected to paper electrophoresis for 40 min. at 4500 volts in Offord's buffer pH 6.4 with a Gilson Electrophorator Model D. The paper was cut into small pieces and counted in Brays scintillation fluid with a Nuclear Chicago Scintillation Spectrometer. Radioactivity of methionine toward the origin at left is not shown. A total of 7,550 cpm were recovered as methionine. Only 0.01 ml from 0.05 ml of rediluted hydrolysate was used. 1.52 mg of recovered myosin was hydrolyzed. Negligible radioactivity is recovered on the anode side of electrophoresis paper. The actual distance between 3-MeHis and TML spots is 20 cms. NGNG dimethylarginine is labelled u-DMA. Sym-DMA is located between u-DMA and 1-MeHis. Methyl ornithine and ornithine migrate faster toward the cathode than lysine.

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pound the unknown obtained from cells labelled with methionine-methyl-^{14}C could be extracted into 0.1 N $Ba(OH)_2$, heated (80°, 45 min.), and the radioactivity (di-methylamine) trapped in dilute H_2SO_4. All of these properties are consistent with those of authentic unsym-DMA.

Unsym-DMA could not be detected in adult muscle myosin whereas the amino acid could be detected even in 1 to 2 mg samples of myosin from cultured muscle cells. It was found that the unsym-DMA content of rat muscle myosin decreases rapidly after birth, just as as 3-MeHis content increased after birth. This rapid decrease in unsym-DMA after birth of rat pups is shown by the data presented in Table I. Eight days after birth, it cannot be detected in myosin hydroly-

Table 1

Basic and Methylated Amino Acids from
Rat Leg Muscle Myosin

Age after birth	Moles per $5x10^5$ gram protein				
	Arg	u-DMA	3-MeHis	His	TML
4 days	208	0.8	+	61	3.0
4	207	0.8	0.1	63	3.8
5	206	0.5	0.5	60	3.4
6	222	0.4	0.3	60	4.4
7	217	0.1	1.1	61	5.0
16	206	(-)	1.0	58	6.1
Adult	222	(-)	1.6	62	4.2
Adult	225	(-)	1.8	62	4.1
*TC (8 day)	205	3.9	+	62	3.5

*TC eight day old preparation of tissue culture from leg muscle cells of neonatal rats.

+ - present in low amount.

(-) not detected. MML was not resolved because of interference from the overload of lysine. Analyses of single preparations are given. The amino acids were measured with a Phoenix analyzer as described in the text.

sates. The greatest amount detected by the analyzer was in myosin isolated from monolayer cultures of rat leg muscle (Table I).

The amount of DMA associated with myosin decreased with the maturity of muscle cells even in culture. Thus, in one experiment, cells in culture were exposed for 24 hours to arginine-guanidino^{14}C* at 9 or 13 days. The cells were scraped in cold saline on days

*Chromatographically pure material of original S.A. 45·7 mCi/mM was used (0.2 μCi/ml in the medium).

10 and 14. Myosin from the cells was isolated (4) and hydrolyzed. The specific activity of the arginine was 41,000 from the younger and 38,200 cpm per mg protein from the older cells, while the respective values for unsym-DMA were 1000 and 465 cpm per mg protein. The ratio of the unsym-DMA:Arg radioactivity decreased from 1:41 to 1:82 in the myosin in this culture over a four day interval. There is no net increase in bulk protein in our monolayer rat muscle cell cultures after 5 to 6 days.

A decrease in unsym-DMA content with age was also noted in myosin isolated from embryonic chick leg muscle. The values decreased from 1.3 residues per 5 x 10^5 gram protein for 7 day embryos to 0.7 for 15 day embryos and it could not be detected in adult chick myosin. The corresponding values for 3-MeHis were (<0.1), 0.3 and 1.5 residues per 5 x 10^5 gram protein.

Unsym-DMA could not be detected in a sample of adult cat soleus muscle myosin obtained from Drs. Adelstein and Kuehl (NHLI-NIH).

Only traces of unsym-DMA were detected in cardiac myosins from rat pups and it could not be detected in cardiac myosins from adult rats or 15 day chick embryos.

Actin preparations from rat and chick muscle showed neither type of DMA on the amino acid analyzer. After exposure of cultured muscle cells to radioactive methionine-methyl-^{14}C, the isolated preparations of actin did not contain radioactive DMA.

The occurrence of unsym-DMA only in tissue culture, embryonic and neonatal skeletal myosins further supports the observations that embryonic or fetal myosins are different from myosins of either adult white or red muscle fibres or cardiac myosins (3,4,13). The variation in methylated lysine and histidine levels may not only be age dependent (Table 1), but also influenced by species, e.g., high methylated lysine content of lobster myosins (4). Myosin methylation

has also been reported to be influenced by specific innervation (13) and by hormonal action (14).

The function of protein methylation is unclear. Analyses of contractile protein methylation may prove useful in tracing the evolution of these proteins.

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