Methylation of canine cardiac myosin in culture

Joan Wikman-Coffelt

Cardiovascular Research Institute, University of California Medical Center, San Francisco (California 94143, USA), 1 October 1979

Summary. Nε,Nε,Nε,trimethyllysine and an unidentified methylated amino acid which co-electrophoresed and co-chromatographed with the hydrolysis product of S-adenosyl-L-methionine, occur in fetal canine cardiac myosin and are isotopically labeled in vitro with S-adenosyl-L-(methyl ^{3}H) methionine between the 10th and the 12th day of culture.

By studying in vitro post-translational modification of myosin it was discovered that while 2 moles of Nε,Nε,Nε,-trimethyllysine per mole of myosin heavy chains are found in all of the mammalian cardiac and skeletal muscle myosins investigated, the occurrence and amount of the other methylated amino acids show a high degree of tissue, species, and developmental variations[1]. The studies described here were undertaken to determine which of the compositional amino acids of myosin are methylated in the intact myocardial fetal cell.

Materials and methods. Canine cardiac cells were cultured and harvested on day 12 as described earlier[2]. Myosin was methylated by adding S-adenosyl L-(methyl ^{3}H) methionine (5 μCi/ml) to the culture medium on day 10. Myosin was purified by a modification of procedures described earlier[3]. The pooled cells were homogenized in 30 ml of 1 mM $MgCl_2$, 0.1 M EGTA, 1 mM DTT, 5 mM ATP, 0.05 M KH_2PO_4, pH 6.8, 10 mM sodium pyrophosphate, and 1 mM sodium azide. After centrifugation (10,000 × g) for 5 min, myosin was extracted from the pellet in 10 ml of the following buffer: 0.1 M KH_2PO_4, 10 mM NaPPi, 0.05 M K_2HPO_4, 0.3 M KCl, 1 mM DTT, and 5 mM ATP. After centrifugation (10,000 × g for 5 min) 10 mg of carrier myosin was added, and the total myosin was recovered in the 35–42% $(NH_4)_2SO_4$ saturation fraction[3]. After precipitating myosin twice with a 9-fold (v/v) dilution of water, the protein was hydrolyzed at 110 °C in 6 N HCl for 24 h in a sealed evacuated tube. For identification of methylated amino acids the samples were 1. electrophoresed in pyridine/acetic acid/water (25:1:225) (v/v) on Whatman No. 54 paper at pH 6.5 for 50 min at 60 V/cm[4], dried and stained with ninhydrin; and 2. chromatographed by descending chromatography (15 h, room temperature) in N-butanol/pyridine/acetic acid/water (15:10:3:12) (v/v)[4].

S-adenosyl-L-methionine (methyl ^{3}H) (11.9 Ci/mole) was obtained from New England Nuclear. L-methionine, Nε,-monomethyllysine, and Nε,Nε,Nε,trimethyllysine were supplied by the Sigma Chemical Company. L-1-methyl and L-3-methyl histidine were supplied by Koch-Light Laboratories, England.

Results and discussion. As in the cell-free studies where post-translational modification of myosin was studied[5], the amino acid Nε,Nε,Nε,trimethyllysine present in cardiac myosin was also labeled in fetal canine cardiac myosin with S-adenosyl-L-(methyl ^{3}H) methionine after 10 days of culturing (figures 1 and 2). This type of specific myosin modification may be used as a marker for the presence of myosin in cultured cells.

Just as the ribosomal protein, L-10, reportedly contains an unidentified methylated amino acid which co-electrophoreses and co-chromatographs with the neutral amino acids[4], so this same methylated amino acid is present in fetal canine cardiac myosin cultured in the presence of S-adenosyl methionine. We wish to report here that this unidentified methylated amino acid appears to be a hydrolysis product of S-adenosyl methionine. When purified S-adenosyl methionine is subjected to the same type of acid

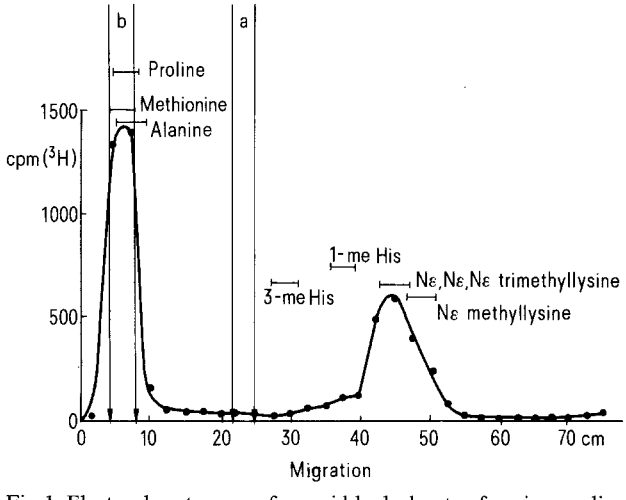

Fig. 1. Electrophoretogram of an acid hydrolysate of canine cardiac fetal myosin labeled with S-adenosyl-L-(methyl ^{3}H) methionine, showing a profile of isotope incorporation, position of ninhydrin stained standards, and of S-adenosyl (methyl ^{3}H) methionine (a) and of the isotopically labeled product of S-adenosyl (methyl ^{3}H) methionine (b). Following electrophoresis the chromatograph was stained with ninhydrin and then marked, cut and analyzed in a scintillation counter for assessing isotope incorporation. S-Adenosyl methionine and the product of S-adenosyl methionine were chromatographed separately and the positions of migration shown by a and b. The 1st peak on the chromatograph corresponds to the hydrolysis product of S-adenosyl methionine and is the unidentified methylated amino acid; the 2nd peak corresponds to the standard, Nε,Nε,Nε, trimethyllysine.

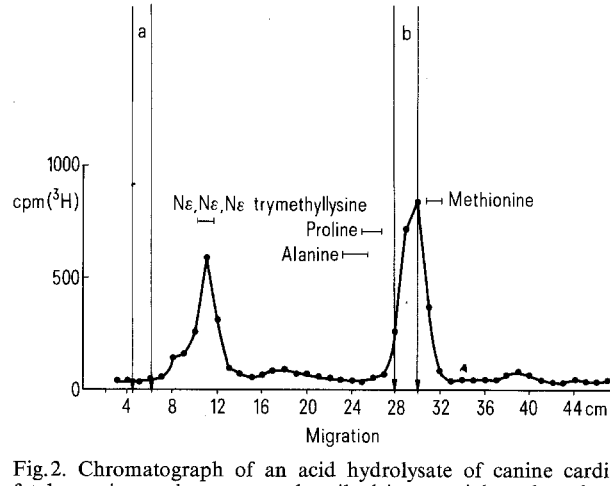

Fig. 2. Chromatograph of an acid hydrolysate of canine cardiac fetal myosin; analyses are as described in materials and methods. The position of S-adenosyl methionine is shown by a and the hydrolysis product of S-adenosyl methionine is shown by b. The 1st peak corresponds to the standard, Nε,Nε,Nε,trimethyllysine and the 2nd peak is the unidentified methylated amino acid corresponding to the hydrolysis product of S-adenosyl methionine.

hydrolysis as used to treat the purified protein, a hydrolysis product appears which co-migrates with the unidentified methylated amino acid (figures 1 and 2). There may be an isotopically labeled product of S-adenosyl methionine which is specifically incorporated into myosin and the L-10 ribosomal protein[4], or S-adenosyl methionine may be a nonspecific contaminant which is then acid-hydrolyzed with the purified protein. The latter seems unlikely since in the case of the L-10 ribosomal protein[4] it is recovered in repeatable stoichiometric amounts[4]. Nevertheless, we believe we have partially identified this methylated amino acid which co-migrates with the neutral amino acids.

We also show here that the methylated amino acid, trimethyllysine, present in myosin, is isotopically labeled following 10 days of tissue culture of fetal canine cardiac cells when myosin has been shown to be present[2].

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Active transport of [^{32}P]thiamine diphosphate in *Escherichia coli*[1]

T. Nishimune[2] and R. Hayashi[3]

Department of Microbiology, Yamaguchi University School of Medicine, Ube, Yamaguchi-ken (Japan 755) and Ohita Medical College, Ohita (Japan 879-56)

Summary. Active uptake of [^{32}P]thiamine diphosphate by *E. coli* was analyzed using an improved method of gel filtration chromatography. The radioactive coenzyme was accumulated without dephosphorylation. From this result it was concluded that thiamine kinase is not involved in the membrane transport of thiamine in *E. coli*.

In the course of our study on the mechanism of thiamine (T) uptake by bacterial cells[4,5], we have noticed that in *Leuconostoc mesenteroides* less than 30% of the actively-accumulated T is phosphorylated[6]. In the active transport of T by *E. coli*, on the other hand, intracellular T is phosphorylated almost quantitatively, and it has been claimed that T kinase is involved in the membrane transport[7,8]. It has also been shown in *E. coli* that thiamine diphosphate (TDP) shares the same route of membrane transport with T. This is based on the following observations; 1. that growth on TDP of a mutant of *E. coli*, which requires TDP for its growth (70-23-107), is competitively inhibited by T[9]; 2. uptake of [^{14}C]T by washed cells of *E. coli* in a specified reaction mixture is inhibited by TDP competitively[10]; and 3., binding of [^{14}C]T by T-binding protein (which is supposed to be an essential component of T uptake system) is shown to be inhibited by about 50% in the presence of 1:1 amount of TDP to [^{14}C]T[4]. If T kinase was involved in T uptake by *E. coli*, TDP should be dephosphorylated before the membrane transport[11]. In this paper we describe evidence that T kinase is not involved in T uptake, using the technique of a gel filtration chromatography[12] for the determination of T phosphates. The determination of T phosphates has been improved, and [^{32}P]TD can be separated from T triphosphate (TTP) and more highly phosphorylated T.

Materials and methods. Synthesis and purification of [^{32}P]TDP. The method described below has been developed and shown to be applicable for the synthesis of [^{32}P]TDP and [^{32}P]TMP. 40 µl of 85% H_3PO_4 was added to 10 mCi of carrier free [^{32}P]H_3PO_4 and heated on a small flame for circa 10 min. 70 mg of P_2O_5 was then added and the mixture kept in a 150 °C oil bath for 10 min. This was then cooled down to 120 °C and 25 mg of T HCl was added to it[13]. The reaction mixture was kept at 120 °C for 15 min and then cooled down to room temperature. 10 ml of 99.5% C_2H_5OH (4 °C) was added and the reaction mixture kept at 4 °C for 2 h. After decantation, the residue was washed again with 10 ml of ethanol and then twice with 10 ml of ethyl ether. The residue was now dissolved in 0.1 ml of 20 mM β-mercaptoethylamine (MEA) at 4 °C and precipitated twice with 10 ml of ethanol. This ethanol precipitation was repeated 2 more times from 0.2 ml of 0.2 N HCl containing MEA. The precipitate obtained was dissolved in 2 ml of 0.3 M potassium phosphate buffer at pH 5.2 containing 5 mM MEA and 1 mM NaCl, and applied to a column of Sephadex G-25 (1.35 × 195 cm) pre-equilibrated with 0.3 M potassium phosphate buffer, pH 7.5, containing 5 mM MEA and 1 mM NaCl. The column was developed using the same buffer at about 15 °C in a dark room, at the rate of 8 g/h. One peak fraction of TDP was selected on the basis of the radioactivity and absorption ($A_{233\ nm}$) maxima of the column eluate. 0.17 ml of 99.5% ethanol was added to each 4-g fraction and the sample stored at −80 °C. A 0.8-ml aliquot of the selected fraction was applied to filter paper (30 cm wide × 60 cm long, Toyo No. 50) which had been soaked in 0.3 M potassium phosphate buffer, pH 5.2, containing 5 mM MEA and 1 mM NaCl (this was also used as the electrode buffer). Electrophoresis was carried out for

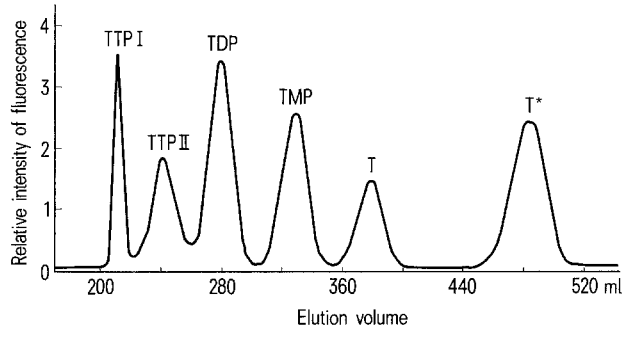

Fig. 1. Separative determination of T and T phosphates on Sephadex G-25. A 1.5-ml sample containing 10 µg of TTP, TDP, TMP, and T was oxidized by BrCN and Nagoh, neutralized and developed as described under 'methods'. The fraction with the maximum fluorescence in TTP I was re-chromatographed in the same position as TTP II. The column was developed at a flow rate of 8 g/h and fractionated into 4-g fractions. Standard thiochrome solutions for the determination of recoveries were prepared at the same time as the sample was prepared, and stored in the dark during the development of the column. * In $K_3Fe(CN)_6$-NaOH oxidation method, this peak is absent.