IN VITRO SYNTHESIS OF LIGHT AND HEAVY POLYPEPTIDE CHAINS OF MYOSIN*

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SUMMARY Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that preparations of highly purified chicken skeletal myosin contain four polypeptide chains: a "heavy" chain (MW about 200,000) and three "light" chains of molecular weights 25,500; 17,600; and 15,200. Three classes of polysomes that differ in size were obtained from homogenates of embryonic chicken leg muscle. The heavy and light chains of myosin are synthesized on two different classes of polysomes from messenger RNA's of different lengths. This suggests that the messages for myosin subunits are monocistronic.

It is currently believed that the myosin molecule consists of two identical "heavy" polypeptide chains each having a molecular weight of about 200,000, and two or three "light" chains having molecular weights of the order of 20,000 (1-7). The light chains appear to be heterogeneous on polyacrylamide gels and cellulose electrophoresis (8-10) and column chromatography (6). They constitute about 10% of the myosin mass (2), and their dissociation from the myosin molecule appears to result in a loss of ATPase activity (2-4, 6,7,11).

Heywood and Rich (12) demonstrated that embryonic chicken muscle homogenate contains polysomes of different sizes. When these polysomes were incubated in vitro with radioactive amino acids and other components of a protein synthetic system, myofibrillar proteins, myosin, actin and tropomyosin, were synthesized. Heywood and Nwagwu (13) recently reported the isolation of a 26S RNA from embryonic muscle polysomes which was assumed to be the messenger for the heavy polypeptide chain of myosin. We have studied the synthesis of the different polypeptide chains of myosin in a cell-free system in order to

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understand the structural and functional relationship of different subunits of myosin. Our results suggest that the large and small subunits of myosin are translated on separate messenger RNA's and are subsequently assembled to form the native myosin molecule.

METHODS

Preparation of Myosin and Polysomes: The preparation and purification of chicken skeletal myosin is described in the legend to Fig. 1. A post-mitochondrial supernatant fraction (PMS) was prepared from tissue homogenates of leg muscles of 14-day-old chick embryos by the method of Heywood et al. (14). Polysomes were prepared from PMS and were fractionated by sucrose density gradient centrifugation into 4 classes, I to IV, by a procedure described

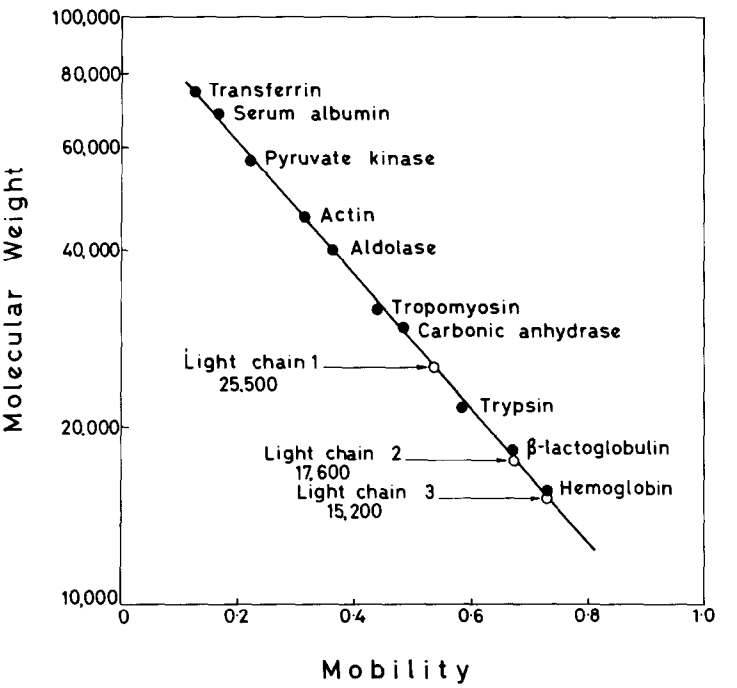

Fig. 1: Determination of molecular weights of light chains of chicken skeletal myosin by SDS-polyacrylamide gel electrophoresis. Myosin was prepared by a modification of the ammonium sulfate method of Tsao (15) as described by Nauss et al. (16). Myosin was further purified by column chromatography on DEAE Sephadex A-50 by the method of Richards et al. (17) using a linear KCl gradient of 0-0.5M KCl in 0.04M K-pyrophosphate, pH 7.4, containing 0.1 mM DTT and 0.1 mM EDTA. Myosin was eluted as a sharp single peak at 0.15M KCl. SDS-gel electrophoresis and calculation of mobilities was carried out using 10 cm 12.5% gels by the method of Weber and Osborn (18).

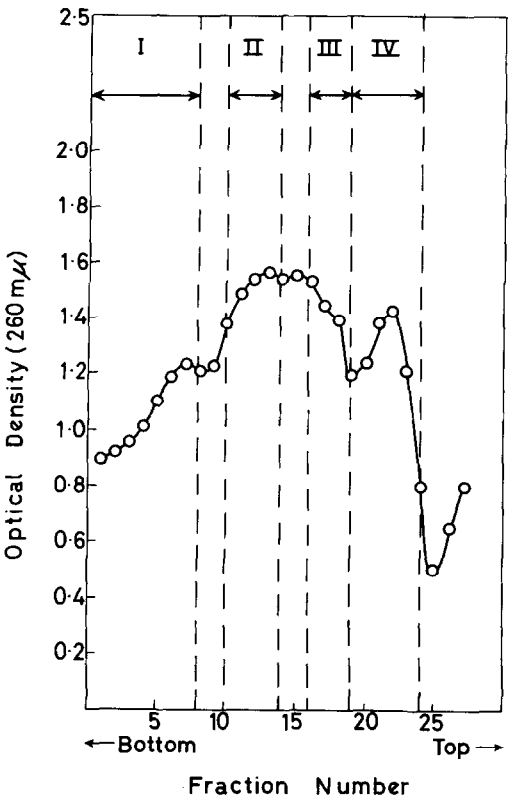

Fig. 2: Sucrose gradient sedimentation pattern of polysomes from homogenates of 14-day-old embryonic chicken leg muscles. Unfractionated polysomes were prepared by layering 5 ml of PMS on a discontinuous sucrose gradient consisting of 15 ml of 40% sucrose and 15 ml of 20% sucrose in buffer II (250 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 10 mM Tris, pH 7.4)as the bottom and top layers respectively. The polysomes were pelleted by centrifuging the gradients at 35,000 rpm for 5 hr. in the International B35 rotor. The pellet was resuspended in buffer II (total polysomes). Thirty-five to forty OD_{260} units of total polysomes were layered on 27 ml of a 15-40% linear sucrose gradient in buffer II. After centrifugation for 2 hours at 25,000 rpm in a Spinco 25.1 rotor at 0°, 1 ml fractions were collected from the bottom of the tube for absorbance measurements at 260 mμ. Four fractions, I to IV, were made from the gradient for in vitro amino acid incorporation. Polysomes from pooled fractions were pelleted by centrifuging at 40,000 rpm in the Spinco 40 rotor for 8 to 12 hr and were gently resuspended in buffer II.

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in the legend to Fig. 2. Fraction I is the class of largest polysomes and IV consists mostly of single ribosomes (Fig. 2). These approximately correspond in size to the four fractions, A to D, described by Heywood et al. (14). Fractions I, II and III contain polysomes with 50-60, 15-25 and 5-9 ribosomes respectively (ref. 12 and our own unpublished results).

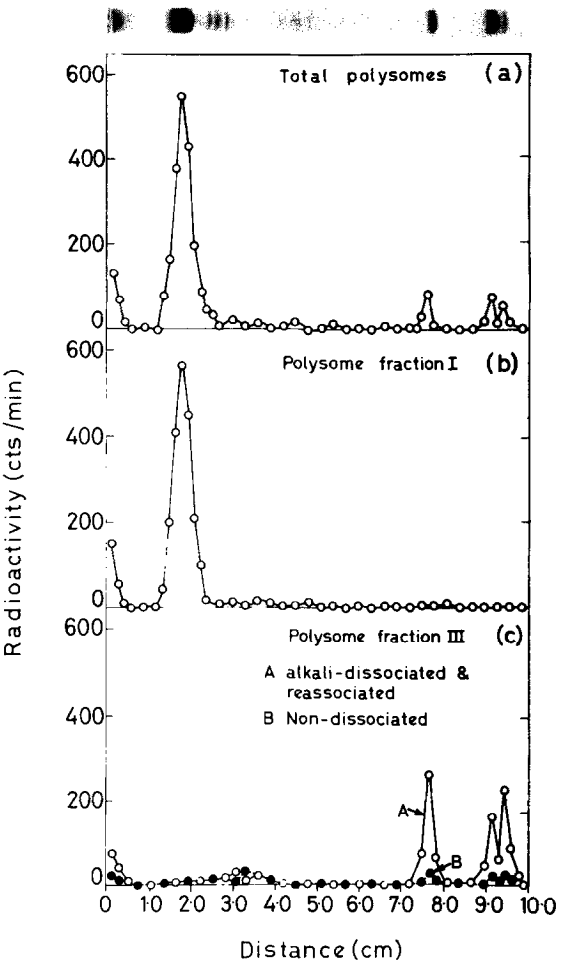

Fig. 3: Distribution of radioactivity in 5% polyacrylamide gels of purified labeled product from in vitro incorporation of polysome fractions. The reaction mixture for in vitro protein synthesis contained in a total volume of 1 ml: 0.15M KCl, 0.02M Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 3 mM DTT, 2 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 µg creatine kinase, 1 µC of a uniformly labeled (^{14}C) amino acid mixture (New England Nuclear Corporation), 0.5 mµmoles each of 5 unlabeled L-amino acids (tryptophane, cysteine, methionine, asparagine and glutamine) not present in the (^{14}C) amino acid mixture, 0.4 mg chicken liver tRNA, 1 mg of pH 5 fraction from embryonic muscle tissue and 1 mg of polysomes (total polysomes in Fig. a, fraction I in Fig. b, and fraction III in Fig. c). In some experiments, instead of the pH 5 fraction, 2 mg of protein of dialyzed 100,000 g supernatant fraction (13) was used. After incubating at 37° for 1 hour (amino acid incorporation was linear for 45-60 minuutes), 50 µg pancreatic RNase was added to terminate the reaction, and a small aliquot was withdrawn to process for hot TCA-insoluble radioactivity (12). The purified product (see Methods) was analyzed using 10 cm 5% gels (18). The method for determination of distribution of radioactivity in the gels is described in the legend to Fig. 4.

In Vitro Amino Acid Incorporation: The conditions of assay are described in the legend to Fig. 3.

Identification of Products of Different Polysome Fractions a) Unfractionated and class I polysomes: Three to 4 mg of carrier myosin was added to the reaction mixtures after amino acid incorporation, and the KCl concentration was adjusted to 0.5M. The solutions were then dialyzed against buffer I (0.5M NaCl, 0.01M sodium-phosphate, pH 7.0, 0.1 mM DTT and 0.1 mM EDTA) containing a large excess of (^{12}C) amino acids. The radioactive product was purified by three cycles of coprecipitation of the carrier myosin at low ionic strength (16). In order to remove any contaminating actin 200 μg of carrier actin were added to the precipitated myosin dissolved in buffer I. Any actomyosin present was dissociated by the addition of 2 mM ATP and 5 mM Mg^{++} and the solution centrifuged at 100,000 g for one hour (16). The supernatant which contained the actin-free myosin was dialyzed against 0.04M $Na_4P_2O_7$ (pH 7.5), 0.1 mM DTT and 0.1 mM EDTA and applied to a 8.0 x 0.5 cm column of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was washed with 10 ml of 0.04M $Na_4P_2O_7$, pH 7.5; under these conditions myosin remains on the column, but other radioactive proteins were eluted. Elution with 0.35M NaCl - 0.04M $Na_4P_2O_7$ (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, released the adsorbed myosin in a sharp peak (2-3 ml). This fraction was concentrated with Aquacide (Cal-biochem.) and dialyzed against buffer I. An aliquot of the dialyzed myosin (70 to 100 μg, 15,000-20,000 cpm/mg protein) was analyzed by SDS[1]-polyacrylamide gel electrophoresis according to the procedure of Weber and Osborn (18) using both 5 and 10% gels. The method for the determination of radioactivity of protein bands in gels is described in the legend to Figure 4.

b) Class III polysomes: After the addition of myosin described above, the mixture was brought to pH 11 with 1N KOH at 0° (4) to dissociate the carrier myosin into heavy and light chains. After 10 minutes at 0°, the pH of the mixture was readjusted to 7.0 to allow reassociation of the polypeptide

[1]Abbreviation used: SDS = sodium dodecyl sulfate

chains. The remainder of the identification procedure, beginning with dialysis against buffer I, was carried out as described above.

RESULTS

Subunits of Chicken Myosin: The electrophoretograms (Figs. 3 and 4) of puri-

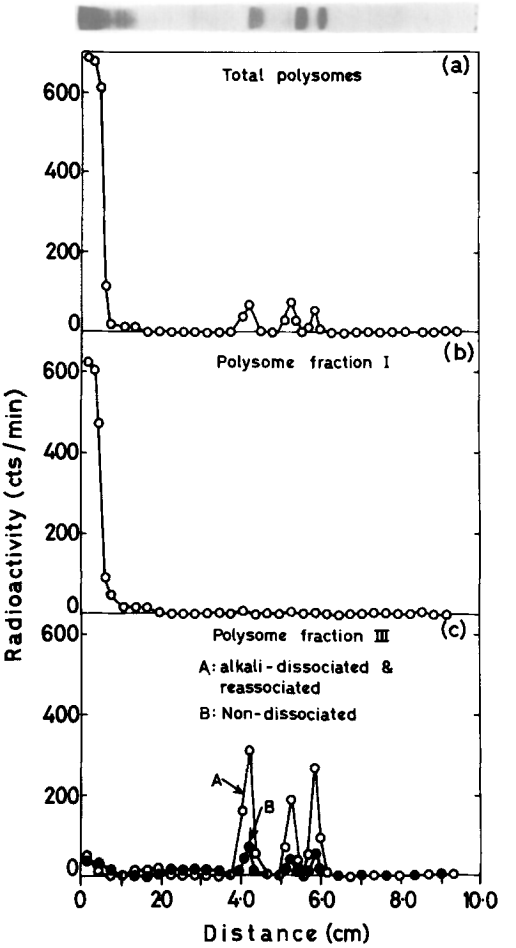

Fig. 4: Distribution of radioactivity in 10% polyacrylamide gels of purified labeled product from in vitro amino acid incorporation of polysome fractions. Methods used for amino acid incorporation and purification of the labeled product were the same as described in the legend to Fig. 3 except that 10 cm length 10% gels were used to analyze the products (18). The gels were stained with Coomassie brilliant blue (18) and sectioned with a Canalco Gel slicer into 1.5 mm slices. Each slice was placed in a counting vial and digested with 100 µl 30% H_2O_2 for 12 hours at 50°. After adding 0.3 ml NCS (Nuclear Chicago) solubilizer and 10 ml of toluene-based scintillation fluid, radioactivity was determined in a Beckman scintillation counter.

fied chicken myosin contained 4 bands indicating the presence of 4 polypeptide chains in myosin. The slowest band corresponds to the heavy chain with a molecular weight of about 200,000 (Fig. 3). Two of the faster moving light chains were not satisfactorily resolved on the 5% gel. When myosin was analyzed on a 10% gel, the heavy chain did not enter the gel. The light chains migrated as three distinct well-resolved bands (Fig. 4). The molecular weights of the three light chains were estimated as 25,500; 17,600; and 15,200 respectively from a plot of log mol. wt. vs. mobility (Fig. 1) using a series of highly purified proteins of known molecular weights as standards.

Synthesis of Myosin Subunits: When unfractionated polysomes were tested for the synthesis of myosin subunits the distribution of radioactivity of the purified product in a 5% gel coincided with the major band of the heavy chain and the three light chain bands (Fig. 3a). The sum of the radioactivity in the three light chain peaks amounted to 13% of the total counts found in the four myosin subunit peaks. On the other hand, with polysome fraction I, all the radioactivity was found in one peak which migrated at the same rate as the heavy chain of myosin (Fig. 3b). With polysome fraction III, radioactivity in the three light chain peaks was detected only when the *in vitro* products were exchanged with unlabeled light chains of the carrier myosin (Fig. 3c, curve A). Control incubations in which the products were not exchanged but were copurified with carrier myosin resulted in a final sample which gave only 5-10 cpm in the regions of the light chains (Fig. 3c, curve B).

These analyses were repeated using 10% gels which gave better resolution of the 17,000 and 15,000 light chains. The results were in agreement with the previous experiments. Again no radioactivity was found in the light chain region when fraction I polysomes were used (Fig. 4b). Most of the radioactivity was found at the top region of the gel where the heavy chain band was located (Fig. 4a and 4b). The synthesis of light chains on fraction III polysomes was demonstrated by an exchange reaction with carrier myosin (Fig. 4c).

DISCUSSION

The results indicate that two classes of polysomes of different sizes

are involved in the translation of mRNA's of different lengths to synthesize the heavy and light chains of myosin. This implies that the messages for myosin subunits are monocistronic. The size of the polysomes (50-60 ribosomes for fraction I and 5-9 ribosomes for fraction III (see ref. 12) is in reasonable agreement with the expected length of the polypeptide chains synthesized on these polysomes. Results obtained with unfractionated polysomes suggest that different subunits of myosin, once synthesized, are assembled in their native configuration to form the complete molecule. However, the light chains made separately with fraction III polysomes are unable to exchange with those of carrier myosin unless the subunits are first dissociated. A large variety of proteins having molecular weights of the order of 20,000-35,000 could be synthesized *in vitro* on polysomes having the size of class III polysomes (12). However, the radioactivity profile and the electrophoretograms of the purified product showed only the presence of heavy and light chains although the carrier myosin was subjected to dissociation during the purification steps. This suggests that the binding between heavy and light chains of myosin is strong and has a high degree of specificity.

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