IN VITRO AMINO ACID INCORPORATION INTO MYOSIN BY FREE POLYSOMES OF RAT SKELETAL MUSCLE

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SUMMARY Rat skeletal muscle polysomes were separated into free and membrane-bound fractions by centrifugation through 2M sucrose. About 80% of total ribosomes extracted were recovered as free polysomes. Sucrose gradient experiments showed similar size distribution patterns for both free and bound polysomes. Chromatographic and electrophoretic analyses of proteins in the cell free amino acid incorporation system indicated that free polysomes are capable of synthesizing myosin.

INTRODUCTION

In mammalian cells, cytoplasmic polysomes are present in at least two forms: one is attached to membraneous structures and the other is free from these membranes (1,2,3,4). It has been shown that the form of polysomes determines the type of proteins which are synthesized on the polysomes (5,6,7). In chick embryo muscle which has little developed membranes of the endoplasmic reticulum, polysomes are unattached to membraneous structure and are capable of synthesizing contractile proteins (8). As muscle cells grow matured, the endoplasmic reticulum becomes prominent and at the same time the amount of membrane bound polysomes are expected to increase. Watts and Reid (3) evaluated the quantity of bound polysomes as 40% of total ribosomes recovered from mouse muscle. In the experiments reported here, free and bound polysomes of skeletal muscle have been tested for their capacity to incorporate amino acids into myosin.

MATERIALS AND METHODS

Preparation of free and bound polysomes. Hind leg muscle from

young rats (60-100g) was removed quickly after animals were decapitated, and separated from outer membranes, connective tissues and nerves. It was then immersed in cold buffer M containing 0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M tris and 0.5 mM EDTA (pH 7.4 at 20°). Approximately 5 g of muscle was cut into small pieces in 5 ml of buffer M, and homogenized in a Sorvall Omni Mixer for 30 sec. at setting 5, then for 1 min. at setting 9. During homogenization the vessel was immersed in an ice water bath. Further grinding was carried out in a glass-Teflon homogenizer (clearance 0.005") by passing the pestle up and down five times as the pestle was rotating at 1,500 r.p.m. In the process of transfer of homogenate from the Omni Mixer vessel to the glass tube, additional 2 ml of buffer M was used for rinsing. The post-mitochondrial supernatant was obtained by centrifuging the homogenate at 12,000 r.p.m. for 20 min. in a SS-34 rotor of a Sorvall RC-2B centrifuge. The free polysomes were obtained by layering 6 ml of post-mitochondrial supernatant over 5 ml of 2 M sucrose in buffer M, and centrifuging at 50,000 r.p.m. for 6-10 hrs. in a Spinco 65 rotor of the L2-65 centrifuge. The resulting pellet was suspended in 0.2-0.3 ml of buffer M, and if necessary, it was stored at -20°. The bound polysomes were recovered from the cloudy zone floating on top of 2 M sucrose at the end of above centrifugation step. This zone was collected and treated with 0.5% Lubrol WX (I.C.I. Organic Inc.) and 1% deoxycholate (Mann Res. Lab. Inc.). It was then centrifuged as in the case of pelleting free polysomes.

Sucrose density gradient. The size distribution of a polysome preparation was observed using a 15-50% linear sucrose gradient centrifugation. The sucrose gradient was made up in buffer M (34 ml), and the polysome sample (0.1-0.2 mg in 0.3 ml buffer M) was layered on top of it. The centrifugation was performed at 27,000 r.p.m. for 2 hrs. in a Spinco SW 27 rotor of L2-65 centrifuge at 0°. Sedimentation patterns

in the gradient was detected with a LKB flow cell of 3 mm light-path attached to a ISCO UV analyser. In this system, the gradient was withdrawn from the bottom of the centrifuge tube using a LKB perpex pump at a rate of 2.5 ml per min.

In vitro amino acid incorporation. The incubation mixture was made in a 0.15 M KCl solution containing 5 mM $MgCl_2$ and 0.02 M tris (pH 7.6 at 20°): each milliliter of the mixture contained 0.5 mg of polysomes, 1 mg of S-150 fraction (10), 3 μ moles of ATP, 0.5 μ moles of mercaploethanol, and 10 mμ mole each of 20 amino acids including 1 μC of ^{14}C-amino acid mixture (NEC-445, New England Nuclear Corp.). Following incubation at 37° for 1 hr. the reaction mixture was made to 0.5 M in KCl, chilled and centrifuged for 2 hr. at 60,000 r.p.m. in a Spinco 65 rotor. To the supernatant, 100 μg of purified rat myosin, and 20 μg of ribonuclease (Sigma Chem. Co.) were added and the mixture was dialysed overnight against a liter of cold 0.02 M KCl - 0.5 mM amino acid mixture at pH 6.8. The resulting precipitate was centrifuged down at 20,000 g in 20 min. and redissolved in 0.5 ml of 0.04 M Na-pyrophosphate (pH 7.5).

Chromatography of myosin. The myosin sample in 0.04 M Na-pyrophosphate was applied on a column (0.9 x 20 cm) of DEAE-Sephadex, A50, equilibriated with the pyrophosphate solution which was also used to elute the non-myosin proteins from the column. This step was completed by elution with 50 ml of 0.04 M Na-pyrophosphate. Myosin was eluted with 0.36 M NaCl - 0.04 M Na-pyrophosphate (pH 7.5).

Acrylamide gel electrophoresis. Electrophoresis of myosin fraction obtained from the above chromatography step was carried out following the procedures including sulfonation described by Paterson and Strohman (11).

Radioactivity assay. The proteins eluted from the DEAE-Sephadex column were precipitated by the addition of trichloroacetic acid to a final concentration of 10% (W/V). The precipitate was collected on a

glass fibre filter, size 2.4 cm (Reeve Angel), washed four times with trichloroacetic acid containing cold amino acids (0.1 mM total), dried in vacuo and counted in a scintillation counter (Beckman LS-100 or -230).

RESULTS AND DISCUSSION

The procedures for preparing polysomes from skeletal muscle have been devised by several workers (12,13,14): the main difference among them is the ionic composition of homogenizing mixture. The ionic strength of the mixture has been shown to affect the yield and the size distribution of recovered polysomes, probably because myosin tends to bind ribosomes (8,14). Watts and Reid (3) measured the amount of free ribosomes as about 60% of the total ribosomes extracted from mouse leg muscle. Their method gave a yield of total ribosomes as about 0.15 mg RNA/gm muscle (wet weight) which is less than one half of that obtained by Chen and Young (14) using rat leg muscle. The latter authors' method for homogenization of muscle was followed in this study. To separate free

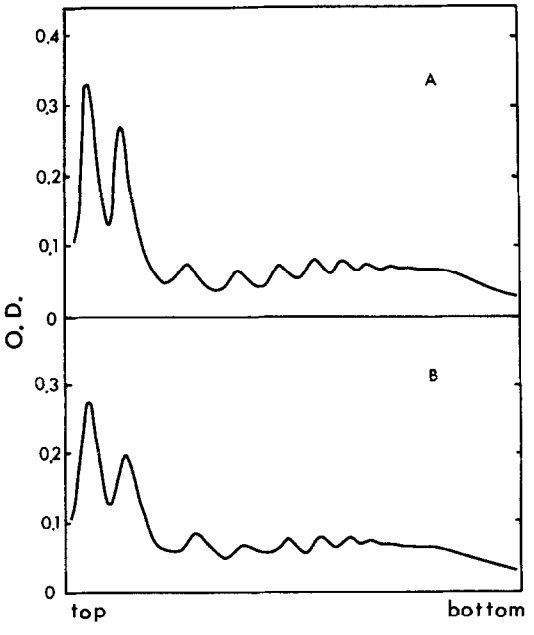

Fig.1 Sucrose gradient profiles of free (A) and bound (B) polysomes. Each type of polysome preparation applied on the gradient contained 0.1 mg of RNA, estimated from the optical density of hot 7% perchloric acid soluble material in the sample.

and bound polysomes, the method of Laga et al. (4) was used. About 80% of total ribosomes were recovered as free polysomes. Although the relative amount of monomer ribosomes in the free polysome fraction appeared slightly larger than that in the bound polysome fraction, polymer distribution patterns were similar for both fractions (Fig.1). There was no detectable effect of detergents on the profile of free polysomes.

Fig.2 shows that free polysomes are active in the incorporation of amino acids into myosin, while bound polysomes appear to label non-myosin proteins rapidly. The proteins eluted from the column with 0.36 M KCl buffer was analysed by acrylamide gel electrophoresis. The method of Paterson and Strohman (11) allows myosin to travel through the gel, whereas other methods show aggregated myosin remaining on top

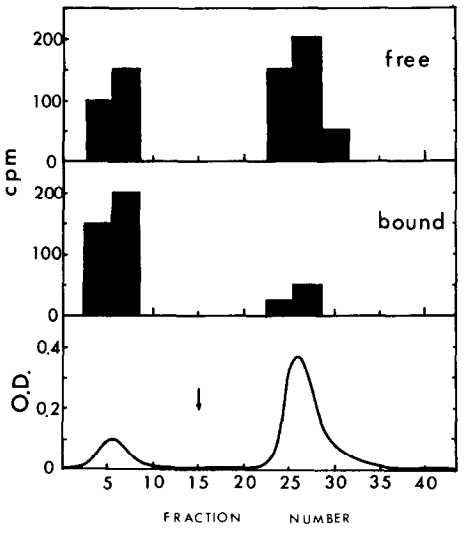

Fig.2 DEAE chromatography of rat myosin added as a carrier to the cell-free amino acid incorporation system. The elution buffer at the start was 0.04 M Na-pyrophosphate (pH 7.5) and at the time indicated by an arrow, changed to 0.36 M KCl - 0.04 M Na-pyrophosphate (pH 7.5). The eluant was collected in fractions of 2.5 ml each. The histograms indicate the radioactivity in the effluent: the samples are from the incubation mixtures with free polysomes (top frame) and with bound polysomes (middle frame). The two types of samples showed the identical elution patterns when monitored by the optical density at 280 nm (bottom frame).

(15). Use of the former method enabled us to see that 80% of the radioactivity associated with the fraction eluted with 0.36 M KCl move with the main chain of myosin in the gel electrophoresis. The activity of the light chain region was too low to be ascertained. Considering the electron microscopic observation by Larson et al. (16) that polysomes are lined up along myofilaments in sarcomeres of skeletal muscle, the above observations infer that the myosin synthesis in mature muscle is carried out on the free polysomes as it is done in chick embryo (8).

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