Purification of fish muscle myosin heavy chain and quantification of the specific polyribosome-bound polypeptide

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1. A purification procedure for fish myosin heavy chain is described. 2. The protein was injected into rabbits for production of antibodies. 3. The specificity of the antibodies was determined by immunoblotting. 4. The enzyme-linked immunosorbent assay technique was applied to quantify myosin heavy chain bound to isolated polyribosomes of epaxial muscle from fish.

INTRODUCTION

Polyribosomes isolated from epaxial muscle of fish have been used to study the rate of protein synthesis *in vitro* (Lied *et al.*, 1982). Ribosome activity changes with feeding conditions, but from determinations of overall activity it is not apparent to what extent a single or a group of muscle proteins is affected (Lied *et al.*, 1982).

To obtain more detailed information on protein-synthesis activity, myosin heavy chain was purified and antibodies against it were produced in rabbits. Available methods for the isolation of the protein from mammalian sources had to be modified because of the unstable nature of the protein and its tendency to aggregate (Connell, 1960). The isolation procedure is described in the present paper, along with verification of the antibody specificity as determined by immunoassays. Myosin heavy chain bound to isolated polyribosomes was quantified by an immunotechnique.

EXPERIMENTAL

Materials

CNBr-activated Sepharose 4B and Sepharose 2B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, ATP, goat anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase, disodium *p*-nitrophenyl phosphate, Fast Blue B and β -naphthyl phosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A., and M_r markers were from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Isolation of muscle myosin heavy chain

Two-year-old cod (*Gadus morhua*) averaging 500 g were caught in the Oster Fjord at the Western coast of Norway. The fish were killed by a blow on the head. Epaxial muscle was dissected, immediately frozen in pulverized solid CO₂ and stored at –80 °C (Lund & von der Decken, 1980; Lied *et al.*, 1982). Myosin was prepared within 4 months of storage.

Muscle of 20 g wet wt. was thawed at 4 °C in sodium phosphate buffer (0.05 M-sodium phosphate, pH 7.5) and passed through a tissue grinder. The minced muscle was suspended in 40 ml of the sodium phosphate buffer and

centrifuged at 4000 *g* for 10 min. This step was repeated twice. The washed tissue was suspended in 5 vol. of ATP/KCl/phosphate buffer (6.7 mM-potassium phosphate buffer, pH 6.0, containing 0.05 M-KCl, 1 mM-MgCl₂ and 5 mM-ATP) stirred slowly, and then centrifuged at 20000 *g* for 10 min. This step was repeated three more times. The final precipitate was suspended in 40 ml of KCl/phosphate buffer (10 mM-sodium phosphate buffer, pH 7.5, containing 0.45 M-KCl, 10.5 mM-containing either 0.015 M-Na₄P₂O₇ and 0.015 M-MgCl₂, or 5 mM-ATP and 1 mM-MgCl₂. Extraction was for 30 min with slow stirring. The suspension was centrifuged at 4000 *g* for 10 min, and the supernatant was then decanted and diluted with 4 vol. of water. The precipitate was immediately collected by centrifugation at 4000 *g* for 10 min. The volume of the precipitate was measured and then the precipitate was dissolved in the KCl/phosphate buffer, at a final ionic strength of 0.35. ATP was added to a final concentration of 5 mM and MgCl₂ to 1 mM, pH 7.5, and then 10 vol. of water was added to precipitate the myosin. The precipitate was dissolved in 5 vol. of 2.4 M-LiCl/0.1 M-KCl/0.13 M-2-mercaptoethanol/2 mM-ATP/0.1 M-glycine, pH 11.35, to dissociate the light chains from the myosin heavy chains. After slow stirring for 1 h at room temperature, the heavy chains were precipitated by adding potassium tricitrate to a final concentration of 0.8 M, and collected by centrifugation at 15000 *g* for 30 min. The precipitate was redissolved in 3 vol. of 0.2 M-imidazole/HCl buffer, pH 6.0, and reprecipitated in 10 vol. of 0.1 M-KCl. The precipitate was dissolved in the KCl/phosphate buffer to give a protein concentration of 2–3 mg/ml. The solution was clarified by centrifugation at 100000 *g* for 1 h. A solution containing urea, EDTA and Tris buffer was added, to give final concentrations 4 M-urea, 10 mM-EDTA, 0.05 M-Tris/HCl buffer, pH 8 (measured at 20 °C), and 0.13 mM-2-mercaptoethanol. Myosin heavy chain was further purified by h.p.l.c. on a TSK 3000SW gel-permeation column and the above urea/EDTA/Tris buffer as eluent. The myosin heavy chain was collected, precipitated by dilution with 10 vol. of water, and redissolved in the KCl/phosphate buffer. The myosin heavy chain was used for production of antibodies. Alternatively, the myosin heavy chains were purified by Sepharose 2B column (2 cm × 25 cm) chromatography with the above urea/EDTA/Tris buffer.

Abbreviation used: e.l.i.s.a., enzyme-linked immunosorbent assay.

Rabbit antiserum

Anti-myosin serum was obtained by immunizing rabbits with intramuscular injections, at weekly intervals, of 300 μg of myosin heavy chain in Freund's complete adjuvant. Serum was collected 5 days after the third and fourth injections.

Isolation of specific antibodies to myosin heavy chain

Myosin heavy chain was coupled to CNBr-activated Sepharose 4B, and antibodies were purified by immunoadsorption (Pharmacia, 1984).

Immunoassay

The e.l.i.s.a. procedure was used (Engvall & Perlmann, 1971). The wells of e.l.i.s.a. microplates were coated with 100 μl of decreasing concentrations of myosin heavy chain, starting with 1 $\mu\text{g}/\text{ml}$. Specific antibodies to myosin heavy chain were added, and the plates were developed with anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase and *p*-nitrophenyl phosphate as substrate. In competitive-inhibition experiments binding of antibody to antigen-coated plates was inhibited by addition of polyribosomes, myosin (control) or unknown samples (Engvall & Perlmann, 1972).

Immunoblotting was carried out as described by Towbin *et al.* (1979), with as primary antibodies those against myosin heavy chain, and as secondary antibodies anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase. Fast Blue B and β -naphthyl phosphate were used to develop the nitrocellulose filters.

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/1% -polyacrylamide gels or gradient gels between 7% and 12% polyacrylamide were run as described by Weber & Osborn (1969) and Laemmli (1970). The marker proteins and their M_r values were myosin (200000), β -galactosidase (116250), phosphor-ylase *b* (92500), bovine serum albumin (66200) and ovalbumin (45000).

Analysis

Proteins were determined by the Coomassie Brilliant Blue method (Bradford, 1976), with serum albumin as a standard. The percentage distribution of proteins in polyacrylamide gels was determined by densitometry.

Preparation of polyribosomes

The preparation procedure for isolation of muscle polyribosomes was that described previously (Lied *et al.*, 1982). It was also used for the isolation of mouse liver polyribosomes.

RESULTS AND DISCUSSION

Purification of myosin heavy chain

The purification procedure applied here differs from others as it includes treatment with various ionic strengths, ultracentrifugation and h.p.l.c. Isolation of myosin from rabbit (Margossian & Lowey, 1982), molluscs (Szent-Györgyi *et al.*, 1971) and cod (Mackie & Connell, 1964) has been described. The difficulty in obtaining pure myosin heavy-chain protein is related to its high affinity for actin. Both ATP and pyrophosphate are required to break the actin-myosin linkage. We applied low-ionic-strength buffer containing ATP

Fig. 1. Polyacrylamide-gel electrophoresis of myosin heavy chain after partial purification and ultracentrifugation

The muscle extract was purified as described in the Experimental section. After removal of the major part of actin the solution was ultracentrifuged to enrich actin polymers in the lower part of the centrifuge tube. Lane 1, before ultracentrifugation; lane 2, upper 50% of the ultracentrifuged solution; lane 3, lower 50% of the ultracentrifuged solution. Actin is indicated by an arrow. The gels were overloaded with protein to make the actin bands visible.

together with repeated washings to remove the major part of actin. Myosin was then brought into solution in high-ionic-strength buffer (1.0.5) and was further purified by reprecipitation at low ionic strength. To keep the myosin in a monomeric form, ATP/MgCl₂ or pyrophosphate/MgCl₂ have to be present (Mackie, 1966). A remaining slight contamination of actin was further decreased by ultracentrifugation, which leads to accumulation of actin polymers in the lower 50% of the centrifuge tube (Fig. 1). Actin as a percentage of total protein amounted to 2% in the upper and 5% in the lower part of the centrifuged solution. The myosin light chains were removed by treatment with urea followed by h.p.l.c. The single peak obtained was myosin heavy chain, as shown by polyacrylamide-gel electrophoresis (Fig. 2).

Throughout the isolation procedure proteins of M_r slightly below that of the major myosin heavy-chain band were seen. Isoforms of myosin heavy chain have been described for rat muscle (Whalen *et al.*, 1981), chicken pectorales muscle (Lowey *et al.*, 1983) and single muscle fibres of humans (Salviati *et al.*, 1984).

Immunological assays

Antibodies to myosin heavy chain were produced in rabbits and their specificity was tested by immunoblotting,

Fig. 2. Immunoblotting of myosin heavy chain

Samples were analysed by polyacrylamide-gel electrophoresis followed by immunoblotting. (a) Polyacrylamide-gel electrophoresis: lane 1, purified myosin heavy chain; lane 2, total muscle extract obtained after centrifugation at 4000 *g* (see the Experimental section); lane 3, markers of M_r 96000, 60000, 46000, 30000, 21000 and 14000. (b) Transfer of proteins to nitrocellulose membranes followed by staining with Amido Black: lane 1, muscle extract as in (a); lane 2, purified myosin heavy chain; lane 3, M_r markers as in (a). (c) Transfer of proteins of nitrocellulose membranes followed by reaction with specific antibodies to myosin heavy chain: lane 1, muscle extract as in (a); lane 2, purified myosin heavy chain.

with anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase as secondary antibodies (Fig. 2). Salt-extracted muscle proteins were compared with purified myosin heavy chain. The specificity of the antibodies is shown by the major band localized at M_r 200000 (Fig. 2c). In a parallel blotting (Fig. 2b) all proteins were stained with Amido Black to confirm transfer of the proteins from the polyacrylamide gels to the nitrocellulose membrane.

The e.l.i.s.a. technique was applied to titrate the antigen. An absorbance above background was registered at antigen concentrations of 1 ng/ml (Fig 3). Inhibition of the myosin-anti-myosin reaction was used to quantify the amount of antigen synthesized on the polyribosomes (Fig. 4). Optimization of the system was worked out with respect to the antigen bound to the solid phase and antibody concentration used to obtain a competitive inhibition by the antigen and unknown samples (Engvall & Perlmann, 1972; Pesce *et al.*, 1978). A myosin concentration of 5.5 μ g/ml (27.5 nM) inhibited the anti-myosin reaction of the myosin-coated plates by 50%. Muscle polyribosomes of intermediate size (fraction I, Fig. 4 inset) gave a 50% inhibition at 60 μ g of ribosomes/ml, which, on the basis of 10.5 absorbance units/mg of ribosomes and an M_r of 4500000, corresponded to 13 nm. The molar ratio of myosin heavy chain to ribosomes became 2:1 instead of the theoretical value of 1:1 (or less than 1:1). Myosin contamination

Fig. 3. Titration of myosin heavy chain by the e.l.i.s.a. technique

Microplates were coated with increasing concentrations of myosin heavy chain and incubated with antiserum followed by incubation with anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase and enzyme substrate.

along the sucrose density gradient during separation of the polyribosomes cannot be excluded. The frequency of the antigen sites along the myosin protein is unknown,

Fig. 4. Competitive inhibition of the myosin-anti-myosin reaction

The ordinate shows the logarithm of the amount of myosin protein added (●—●) or the logarithm of the polyribosome concentration, assuming that 10.5 absorbance units at 260 nm are equal to 1 mg of ribosomes/ml: ○—○, fraction I; △—△, fraction II of the muscle polyribosomes (shown in the inset); ●—●, liver polyribosomes. The microplates were coated with 0.1 µg of myosin heavy chain followed by incubation with myosin heavy chain or polyribosomes at the concentrations indicated. Antiserum to myosin heavy chain was added followed by the anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase and enzyme substrate. The results are expressed as absorbance as a percentage of maximal reaction.

but should increase with the length of the molecule. Chain length increases with the size of the polyribosomes (Lodish, 1976), and the results give a statistical average concentration of the myosin heavy chain synthesized. When heavy polyribosomes with s_{20} sedimentation values of more than 1000 S were analysed for myosin (fraction II, Fig. 4 inset) a 50% inhibition was obtained at 32 µg (7 nm). This results in a ratio of myosin to ribosomes of 3.9:1, thus confirming that a greater number of molecules and/or antigen sites were available. Similar results were obtained by direct titration of polyribosomes with myosin antibodies (results not shown here). Mouse liver polyribosomes were used at the same ribosome concentrations as muscle polyribosomes; they showed a low inhibition at ribosome concentrations of more than 100 µg/ml (Fig. 4).

The curves obtained with myosin bound to polyribosomes were not strictly parallel to the standard curve. Subtraction of the values obtained with liver polyribosomes will shift the curves at the values obtained below 50% inhibition towards a more parallel appearance. One reason for the non-parallel behaviour of the standard and the unknown sample would be that purified myosin is compared with the growing polypeptide chains of myosin. Exposure of sites may differ, especially if folding of the growing polypeptides was not completed. The equilibrium between antigen and antibody will then shift (Pesce *et al.*, 1978; Belanger & Masseyeff, 1982). The higher the affinity, the less antigen is required to achieve a 50% binding.

In conclusion, the procedure described for isolation of fish muscle myosin heavy chain resulted in a highly purified protein preparation. The antibodies produced in rabbits were specific for the myosin heavy chain, and they were used to quantify its synthesis on isolated polyribosomes from epaxial muscle of fish.

The work was supported by a grant from the Norwegian Fisheries Research Council and a fellowship to E. L. from Nordic Research Course.

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