CELL-FREE SYNTHESIS OF MYOSIN BY CARDIAC MYOFIBRILLAR RIBOSOMES

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SUMMARY Ribosomes from rat cardiac muscle myofibrils are shown to be capable of synthesizing myosin in vitro. Chromatographic analysis of proteolytic digests of myosin formed in vitro and that synthesized in vivo indicates that the myosin synthesized in the cell-free system is identical to the native molecule.

INTRODUCTION

On fractionation of cell components the bulk of RNA in muscles sediments with the heavy fraction containing myofibrils and nuclei unlike in other types of cells where microsomes are richest in RNA (1,2,3). A major proportion of this RNA is firmly bound to the "myofibrillar structure" in the form of ribosomes (4). While the exact location of these ribosomes within the myofibril is not yet established indications are that they are in association with the myosin filaments (5). In a recent study we have demonstrated the capacity of rat cardiac myofibrils to synthesize protein, both in vivo and in vitro (3). The data presented in this report suggest strongly that cardiac myofibrillar ribosomes of rats are able to synthesize myosin, the most abundant structural protein of muscles, in vitro.

MATERIALS AND METHODS

Preparation of myosin. Male Wistar rats weighing 120-150g were injected intraperitoneally with ^{3}H-leucine (0.2 µCi/g body weight, spec. act. 7600 mCi/mmole; obtained from the Isotope Division, Bhabha Atomic Research Centre, Bombay)

for in vivo labelling of myosin. The animals were sacrificed by cervical dislocation 2 h after injection. Myosin, thus labelled, was isolated and purified employing the method of Fink (6). Unlabelled myosin was prepared from cardiac muscle of rats following the same method.

Preparation of myofibrillar ribosomes and in vitro amino acid incorporation. Myofibrillar ribosomes were prepared from rat cardiac muscle and were utilized for cell-free protein synthesis as described in a previous publication (3). The labelled precursor used was ^{14}C-leucine (2 µCi/single assay system, spec. act. 50.1 mCi/mmole; obtained from the Isotope Division, Bhabha Atomic Research Centre, Bombay). The total fluid volume of the incubation mixture was 1 ml. Ribosomes and cell sap amounted to 2 mg protein and 600 µg protein respectively. After 1 h incubation in a shaking water bath at 37°C the tubes were chilled in ice and 10 mg unlabelled carrier myosin, in 4 ml buffer containing 0.5 M KCl, 0.01 M tris (pH 7.4) and 0.001 M EDTA, was added to each assay system. The contents of four tubes were combined and myosin was re-isolated and purified at 4°C by various step-wise treatments, in two cycles, following the method described by Heywood and Rich (7). In this procedure, the isolation and purification of myosin have been achieved by taking advantage of its solubility in high ionic strength KCl. Myosin is insoluble in solutions of low ionic concentration of the salt. In addition, myosin precipitates over a narrow (40-47%) concentration range of $(NH_4)_2SO_4$. At the end of each step, aliquots were taken and the protein was precipitated with 5% trichloroacetic acid (7).

Determination of protein and assay of radioactivity. Protein was estimated by the method of Lowry et al. (8).

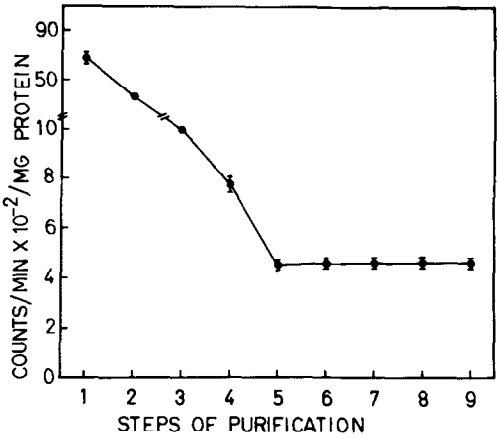

Fig.1. Specific activity of myosin, synthesized in the cell-free system, obtained at the end of sequential purification steps (1 to 9). Values are mean ± SE of 3 experiments and points without bars include SE. For details of purification steps see ref. 7.

Radioassay was carried out in a Beckman LS-100 liquid scintillation spectrometer. The specific activity of protein was computed.

Chromatographic analysis of proteolytic digests of myosin. Approximately 7 mg each of in vivo ^{3}H-leucine-labelled myosin and in vitro ^{14}C-leucine-labelled myosin were mixed in KCl buffer. After overnight dialysis against 1% NH_4HCO_3, the resulting suspension (pH 8.3) was used to prepare a proteolytic digest by subjecting to the action of trypsin and chymotrypsin following the method of Rourke and Heywood (9). The peptide mixture, in 1.5 ml 0.5 M KCl, was applied to a Sephadex G-50 column (1 x 25 cm). The peptides were eluted with 120 ml 0.5 M KCl. Fractions (4 ml) were collected, and optical density (at 280 nm) and radioactivity (^{3}H and ^{14}C) were measured.

RESULTS AND DISCUSSION

The results presented in Fig. 1 show that myosin isolated from among the products of the cell-free amino acid incorpora-

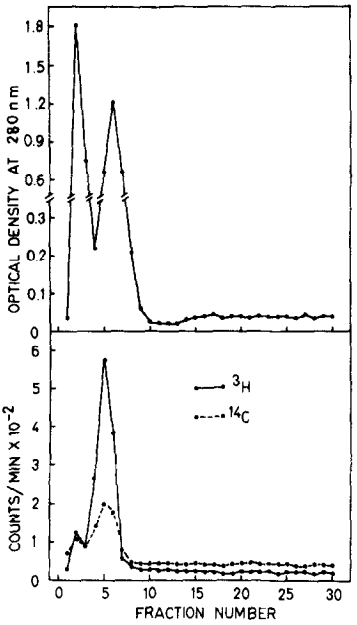

Fig.2. Chromatographic separation of proteolytic digest of myosin, ^{3}H-leucine-labelled in vivo and ^{14}C-leucine-labelled in vitro, on Sephadex G-50 column.

tion system is appreciably labelled. The specific activity of protein is reduced considerably during the initial steps of purification. Nevertheless, after the final step (step 5) of $(NH_4)_2SO_4$ precipitation in the first cycle there is no further change in specific activity. Thus, during the second cycle of purification (steps 6-9) the specific activity remains constant. If the native protein can be continually re-isolated at constant specific activity it would indicate that the particular protein has been synthesized in the cell-free system. In the present experiments, the specific activity of myosin remained constant despite repeated re-isolation suggesting that the newly synthesized protein has the same chemical properties as the unlabelled carrier myosin added to the cell-free system. This further implies that polypeptide chains of myosin were assembled and then assumed native

configurations, including subunit associations, since the individual subunits do not have the same chemical properties as the native molecule. A similar experimental approach has been made use of by Heywood and Rich (7) for demonstrating the in vitro synthesis of specific myofibrillar proteins by different polysome fractions derived from embryonic chick muscles. Their results have indicated that the large subunit of myosin (mol. wt. 200,000) is synthesized by polysomes containing 50-60 ribosomes. More recently, Sarkar and Cooke (10) have shown the in vitro synthesis of light and heavy chains of myosin on separate messenger RNAs and their subsequent assembly to form the native molecule. Low et al. (11) have also identified separate polysomes active in the synthesis of light and heavy chains of myosin. That free polysomes, but not membrane-bound polysomes, synthesize myosin in vitro has been shown in the case of rat skeletal muscle (12). It must be pointed out that in all these studies the intracellular site of origin of polyribosomes was not identified. The polysome fractions were derived from muscle homogenates prepared in buffer solutions of ionic strength sufficiently high to solubilize myosin, with which myofibrillar ribosomes are probably associated (5). Since bulk of RNA in muscle is associated with myofibrils in the form of ribosomes (3,4) a considerable proportion of polyribosomes used in those studies were presumably of myofibrillar origin.

The specific activity of myosin re-isolated from the cell-free system is only a fraction of the specific activity of the initial mixture. Since the ribosomes used in our studies comprised of total myofibrillar ribosomes, it may be inferred that myofibrillar proteins other than myosin might have also

been synthesized in this system, perhaps at a rate faster than that of myosin synthesis. This inference is consistent with recent observations that comparatively low molecular weight myofibrillar proteins such as troponin, α-actinin, tropomyosin and actin are synthesized and turned over more rapidly than myosin (13,14).

Seeking additional evidence for the authenticity of myosin synthesized in vitro, we have compared the proteolytic digests of myosin synthesized in vivo and in vitro. The technique employed yielded a limited resolution of the peptides (Fig. 2). Nevertheless, it provides a reasonably good comparison of myosin synthesized in vivo with that formed in the cell-free system. The radioactivity is associated with the peptides. Further, the radioactivity peaks in both cases appeared in the same fractions. However, the amount of radioactivity and peptides in the two were not proportional. This is not inexplicable because the myosin molecule, though relatively rich in leucine (the radioactive amino acid used in our studies), is richer in other amino acids such as glutamic acid, lysine and aspartic acid (14).

The present findings suggest that myosin (and probably other contractile proteins) is synthesized by ribosomes present on myofibrils.

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