Posttranslational Incorporation of Contractile Proteins into Myofibrils in a Cell-free System

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Abstract. The incorporation of newly synthesized protein into myofibrils has been examined in a cell-free system. Myofibrils were added to a reticulocyte lysate after the in vitro translation of muscle-specific poly(A)$^+$RNA. Only a small number of the many synthesized proteins were found to associate with the exogenously added myofibrils. These proteins were all identified as sarcomeric components and had subunit mobilities (M_r) of 200, 140, 95, 86, 43, 38, 35, 25, 23, 20, and 18 kD. The association was rapid ($t_{1/2} < 15$ min) and, for most of the proteins, relatively temperature insensitive. Except for a 43-kD polypeptide, tentatively identified as β-actin, none of the proteins encoded by brain poly(A)$^+$RNA associated with the myofibrils. When filaments made from purified myosin or actin were used as the "capture" substrates, only thick or thin filament proteins, respectively, were incorporated. Incorporation was substantially reduced when cross-linked myosin filaments were used. These results are compatible with a model in which proteins of the sarcomere are in kinetic equilibrium with homologous proteins in a soluble pool.

THE myofibril of striated muscle has long served as a model system for the study of cytoskeletal proteins and their functions. Traditionally, this organelle has been considered a relatively inert contractile machine differing in character from the more dynamic cytoskeleton of nonmuscle cells. Recent studies on muscle development and the compositional remodeling of myofibrils which accompanies this process make such a traditional view less tenable (McKenna et al., 1985*b*; Saad et al., 1986*b*; Mittal et al., 1987; reviewed by Fischman, 1986). It is conceivable that myofibrils are dynamic organelles more similar to the microtubules and microfilaments (Kirschner and Mitchison, 1986; Korn, 1982; Pollard and Cooper, 1986) of nonmuscle cells than previously suspected.

Remodeling of myofibrils occurs throughout life and must take place in the context of ongoing muscle contraction. Skeletal muscle is the major amino acid store of the vertebrate body and atrophy of muscle is an obvious feature of starvation (Cahill, 1970; Goldberg and Dice, 1974). Furthermore, hypertrophic and atrophic changes of muscle cells are known to correlate with the functional demands placed on this tissue (Goldspink, 1983). Recent studies have also shown that the expression of contractile protein isoforms within individual muscles changes predictably during the course of muscle development and regeneration (Fischman, 1982; Bandman, 1985; Obinata, 1985; Saad et al., 1987). In short, mechanisms must exist for the insertion and removal of proteins from myofibrils in a manner which does not impair the contractile functions of the whole cell.

All of the myofibrillar proteins turn over with characteristic half-lives (Zak et al., 1979) and virtually all exhibit first-order turnover kinetics (Martin et al., 1977; Zak et al., 1977; Martin, 1981; Millward and Bates, 1981; Crisona and Strohman, 1983). In addition, individual protein subunits within a thick or thin myofilament can exhibit different decay rates (Silver and Etlinger, 1985), suggesting that removal of these proteins cannot be accounted for by en bloc elimination of whole sarcomeres or myofilaments. Current results seem most compatible with a degradative process involving the individual proteins or their subunits, possibly in a monomer pool that is in dynamic equilibrium with polymeric elements in the myofibrils.

Despite the large body of biochemical information on the contractile proteins, there remains a significant gap between our understanding of in vitro filament polymerization reactions (Oosawa, 1983; Pepe, 1983) and the in vivo processes involved in filament assembly and sarcomere remodeling. In this report, we present an experimental system that offers promise for examining the posttranslational steps of protein insertion and removal from cytoskeletal structures. Newly synthesized myofibrillar proteins associate at high affinity with myofibrils in a reticulocyte lysate system. This process is tissue and filament specific, rapid, and temperature independent. The data are compatible with a model in which the contractile proteins of the myofilaments are in equilibrium with a soluble pool of monomers and suggest that compositional changes of the sarcomere rapidly reflect mRNA complexity within muscle cytoplasm.

Portions of this work have appeared previously in abstract form (1986. *Biophys. J.* 49[No. 2, Pt. 2]:233*a*; 1986. *J. Cell Biol.* 101[No. 5, Pt. 2]:33*a*).

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Materials and Methods

Preparation of mRNA

Total RNA was isolated from the pectoralis major muscles of 19-d-old chick embryos by either the guanidinium/cesium chloride procedure (Glisin et al., 1974; Ullrich et al., 1977) described by Maniatis et al. (1982) or by the LiCl/urea method of Auffray and Rougeon (1980). Poly(A)$^+$RNA was isolated from total RNA by oligo(dT)-cellulose column chromatography according to the method given in Maniatis et al. (1982).

In Vitro Translation

Translations were performed as outlined in Maniatis et al. (1982) in 25–50-μl translation mixtures. Reticulocyte lysates were prepared by a modification of the method of Pelham and Jackson (1976) and Jackson and Hunt (1983) as described by Reinach and Fischman (1985). [^{35}S]Methionine was purchased from New England Nuclear (Boston, MA). Radioisotope concentrations were adjusted to provide ~100 μCi/25 μl of translation mixture. Lysates were supplemented with an ATP-generating system containing creatine kinase and creatine phosphate at final concentrations of 0.25 mg/ml and 10 mM, respectively.

Preparation of Myofibrils

Muscle fiber bundles, from the pectoralis major muscle of adult White Leghorn chickens, were stretched, tied to wooden dowels, and extracted for 12 h at 4°C in myofibril buffer (0.1 M KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 10 mM KPO_4, pH 7.0, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.5% Triton X-100. The Triton X-100 was then removed and the muscle stored at −20°C in 50% glycerol, 10 mM PO_4 (pH 6.8), and 2 mM EGTA. Just before use, the bundles were cut into 1-2-mm^3 pieces, homogenized in an Omnimixer (Sorvall Instruments, Newton, CT) at half speed for 30 s on ice, and washed three times in myofibril buffer. By phase-contrast microscopy, the vast majority of myofibrils were seen to contain ~2.3-μm-long sarcomeres with well-defined A-, I-, and Z-bands.

Preparation of Synthetic Myosin and Actin Filaments

Column-purified pectoralis myosin and synthetic myosin filaments were prepared by dialysis as described by Saad et al. (1986*a*, *b*). F-actin, extracted and purified from the back muscles of adult rabbits by the method of Spudich and Watt (1971), as modified by Pardee and Spudich (1982), was a gift of Dr. Joel Pardee. Before use in the posttranslational incorporation assays, filament suspensions were centrifuged at 12,000 *g* for 3 min. The protein concentrations of these suspensions were measured and adjusted appropriately. Negatively stained samples of both types of filaments were examined by transmission EM and each exhibited the structural features characteristic of the respective proteins (Huxley, 1963). Synthetic myosin filaments were cross-linked using 10 mM 1-ethyl-3-3-dimethylamino propyl carbodiimide in low salt buffer (0.125 M KCl, 10 mM imidazole, pH 6.8) at a protein concentration from 1 to 2 mg/ml. Cross-linking was performed at room temperature for 2 h with gentle rotation. The reaction was stopped by dialysis against low salt buffer with 1 mM DTT.

Electrophoresis

One-dimensional SDS gel electrophoresis was performed according to the procedure of Laemmli (1970). Typically, minigels (Mikawa et al., 1981) were used with a 3% stacking gel over a 5–15% linear gradient of acrylamide in the separating gel. When gradient gels were not used, the alternative system has been specified in the appropriate figure legend. Standard, 16 × 16 × 0.075 cm, 5–20% gradient gels with 3% stacking gels were used when the amount of radiolabel in specific polypeptide bands was quantitated by scintillation counting. Two-dimensional gel electrophoresis was performed by the methods of O'Farrell (1975) and O'Farrell et al. (1977) as modified by Mikawa et al. (1981). After electrophoresis, gels were stained with 0.4% Coomassie Brilliant Blue in 50% ethanol, 7% acetic acid, and destained in 7% acetic acid. Large formate gels were prefixed in 12.5% TCA (J. T. Baker Chemical Co., Phillipsburg, NJ) followed by 10% acetic acid before staining. After immersion in EN^{3}HANCE (New England Nuclear) for 30–60 min and water for 30 min, the gels were dried under vacuum (Slab Gel Dryer, model 1125B; Bio-Rad Laboratories, Cambridge, MA). Exposure to Kodak X-AR5 film was performed at −70°C.

Quantitation

Scanning densitometry was performed on fluorograms of EN^{3}HANCE-treated minigels with a Transmittance/Reflectance Densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, CA). Data were analyzed on an IBM XT/PC (IBM Instruments Inc., Danbury, CT) using the GS350 software program provided by Hoefer. All scans were repeated at least three times with three exposures of each gel to assure that film densities were in the linear range of the emulsion gamma curve. Thus, silver-grain density was directly proportional to the content of radioisotope within the underlying gel. The background densities from film regions overlying the gels but lacking radioactive protein were subtracted from the density values recorded over the samples that had been subjected to electrophoresis.

The amount of radiolabel incorporated into specific polypeptides was also established by scintillation spectrometry. The fluorogram of an EN^{3}HANCE-treated gel was aligned over the dried gel and those polypeptides which were incorporated into the myofibrils were marked on the gel by passing a dissecting needle through the film into the sample. These bands, and comigrating bands in lanes loaded with samples of the total lysate, were excised from the gel with a razor blade. Once excised, the gel bands were placed in scintillation vials containing 10 ml of Fluorosol (National Diagnostics, Inc., Somerville, NJ). Samples were vortexed, heated to 50°C for 3 h, cooled, vortexed again, and counted in a Tri-Carb 300 Scintillation Spectrometer (Packard Instrument Co., Inc., Downer's Grove, IL). Separate background determinations were made for regions of the gel which had been loaded with total lysate and myofibrillar samples. Once the radioactive content of any particular band had been established, the gram content of newly synthesized polypeptide was calculated based on the specific activity of the radiolabel, the number of methionines in the molecule, and the molecular mass of the polypeptide. The methionine values for each protein were taken from the published literature. Values for chicken muscle proteins were used when available; otherwise, mammalian proteins were selected. The literature cited has been noted in the footnotes for Table I.

Determination of Acid-precipitable Radioactivity

Cold TCA-precipitable counts were measured by the method of Jackson and Hunt (1983). Samples were counted in 5 ml of Aquassure (New England Nuclear). All values were corrected for background and were adjusted for the volume of the initial sample.

Protein Determination

All protein determinations were performed by the Bradford assay (Bradford, 1976).

The Assay System

Poly(A)$^+$RNA from pectoralis muscle of 19-d-old chick embryos was incubated for 30 min at 37°C in a reticulocyte lysate in the presence of [^{35}S]methionine. The translation reactions were terminated by immersion in ice or in some cases by the addition of RNase A to a final concentration of 1 μg/ml. Each translation reaction was then made 0.1% in both Triton X-100 and BSA, clarified by centrifugation (12,000 *g*) for 5 min, and incubated for 1 h in the presence of from 0 to 2 mg/ml myofibrils isolated from the pectoralis muscle of adult chickens. The myofibrils, with any associated labeled protein, were then separated from the supernatant by centrifugation (12,000 *g* for 5 min), washed once in myofibril buffer, and the samples prepared for SDS-PAGE and fluorography. In the quantitation experiments, lysates were brought to 10 mM EGTA and supplemented with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml E64, and 1 μg/ml bestatin before the myofibrils were added.

Results

As shown previously (Whalen et al., 1979; Devlin and Emerson, 1978; Garrels, 1979; Mikawa et al., 1981), a wide range of polypeptides with M_rs of >200 kD to <15 kD are encoded by the mRNA isolated from 19-d-old embryonic chick muscle (Fig. 1 *A*, lane *a*). Virtually none of these proteins sedimented at 12,000 *g* in the absence of added myofibrils after a 1-h incubation on ice (compare lanes *b* and *c*). The electrophoretic pattern of these samples did not

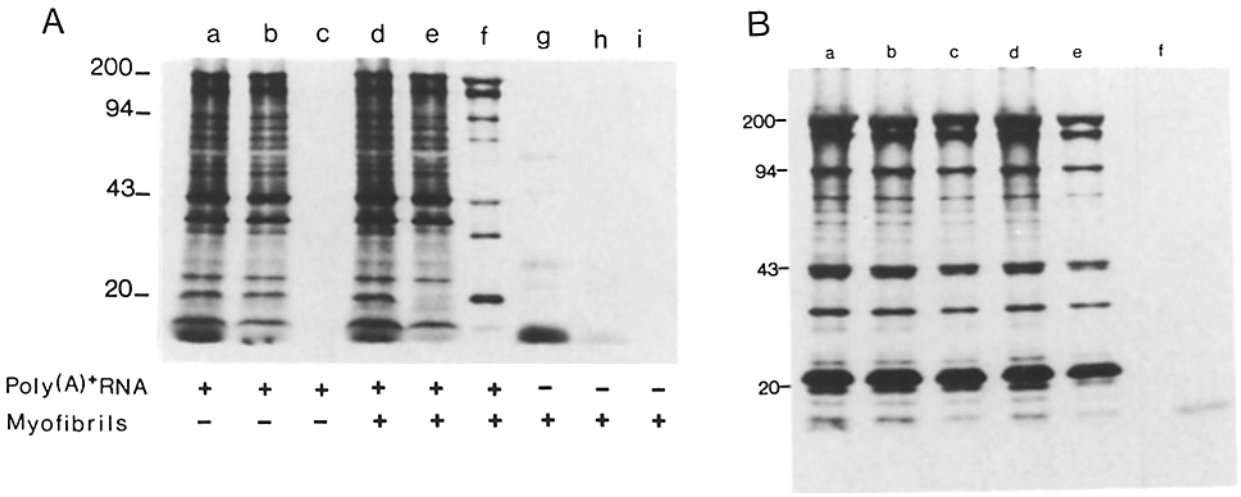


Figure 1. Fluorogram of those labeled proteins that cosediment with myofibrils. (*A*) Poly(A)$^+$RNA from the pectoralis muscle of 19-d-old chick embryos was translated in a reticulocyte lysate system in the presence of [^{35}S]methionine. Samples were cooled to 4°C, made 0.1% in both Triton X-100 and BSA, and centrifuged at 12,000 *g* for 5 min. The pellet was discarded and an aliquot of the supernatant was saved for electrophoresis. Myofibrils from adult pectoralis muscle were added to the remaining supernatant (to give 1 mg/ml) and incubated for 1 h on ice. After centrifugation (12,000 *g* for 5 min), the pellet was washed once in myofibril buffer and dissolved in a volume equal to the original supernatant with 9.5 M urea. Aliquots equal to 1/50 the volume of the initial supernatant fraction and 1/10 of the myofibril pellet were separated by SDS-PAGE. Dried EN^{3}HANCE-treated gels were exposed to Kodak X-AR5 film for 15 h. Lane *a*, total translation products in the initial supernatant fraction before myofibril addition; lane *b*, translation products remaining in the supernatant fraction after addition of myofibril buffer and centrifugation; lane *c*, translation products which pelleted from the sample in lane *a*; lane *d*, labeled proteins in the lysate before myofibril addition; lane *e*, labeled proteins in the lysate after incubation for 1 h with myofibrils; lane *f*, labeled proteins in the myofibril pellet after incubation in the lysate for 1 h; lane *g*, the reticulocyte lysate after translation but without addition of any poly(A)$^+$RNA; lane *h*, the supernatant fraction of the same sample in lane *g* but after incubation with myofibrils for 1 h and their removal by centrifugation; and lane *i*, the myofibril pellet from the same sample as in lane *g*. (*B*) Fluorogram of incubated myofibrillar pellets after one to five washes in myofibril buffer (lanes *a–e*, respectively). Lane *f*, labeled proteins which cosedimented with the myofibrillar pellet after a 1-h incubation in a reticulocyte lysate containing 100 μg/ml cycloheximide.

change over the course of this incubation with or without the addition of protease inhibitors (e.g., compare lanes *a* and *b*). After centrifugation in the presence of myofibrils, 10 major radioactive protein subunits were detected reproducibly in the pellet with M_rs of 200, 140, 95, 86, 43, 38, 35, 25, 20, and 18 kD (lane *f*). By coelectrophoresis with isolated myofibrils or protein standards (results not shown), it was established that these are the major sarcomeric protein subunits (S-proteins)[1] of the myofibril. Not all of these proteins are clearly resolved in Fig. 1 *A*; with longer fluorographic exposure or two-dimensional gels, however, all of these bands were evident. All of the proteins which cosedimented with the myofibrils were encoded by the added mRNA (lanes *g–i*). Cycloheximide (100 μg/ml) blocked the synthesis of these proteins (Fig. 1 *B*, lane *f*). None of the S-proteins were eluted from the myofibrils after as many as five washes in myofibril buffer (Fig. 1 *B*, lanes *a–d*).

The cosedimentation of labeled proteins with the myofibrils resulted in a loss of proteins of equivalent mobility from the supernatant fraction (compare lanes *d*, *e*, and *f* of Fig. 1 *A*). Addition of fresh myofibrils to this supernatant fraction led to the additional capture of some S-proteins (Fig. 2). The amount of each protein captured in this second incorporation reaction was not necessarily equivalent to that in the first. For example, very little troponin-C incorporated in a second incubation, whereas the incorporation of actin and tropomyosin was approximately equal in both. Still less incorporation was found after a third round of incubation with myofibrils (Fig. 2, lane *c*).

Two-dimensional gel electrophoresis substantiated and clarified the results obtained by one-dimensional analysis. This was particularly evident for a cluster of polypeptides characteristic of the troponins and myosin light chains (Fig. 3). One polypeptide, for example, a protein of 23 kD that comigrated with troponin-I was clearly apparent on two-dimensional gels (Fig. 3) but only poorly resolved by one-dimensional electrophoresis.

These results indicate that a group of protein subunits with molecular masses characteristic of the major sarcomeric components will associate with added myofibrils posttranslationally and do so in a cell-free system. The following experiments were designed to test the specificity and quantitative nature of this reaction.

Quantitation of Protein Association

The association of S-proteins with the myofibrils was quantified by establishing the amount of radiolabel incorporated into each S-protein band, as well as the amount present in those bands which comigrated with the S-proteins in total lysate samples. From these data we calculated the grams of each protein subunit that were synthesized in the lysate and how much of each cosedimented with the myofibrils. The results of six experiments are presented in Table I. (We suspect that some of the variability in these numbers may have

1. *Abbreviation used in this paper*: S-protein, sarcomeric protein subunit.

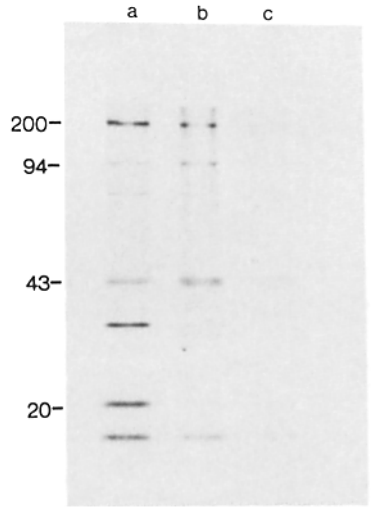


Figure 2. Examination of repetitive capture. Myofibrils were incubated in a reticulocyte lysate after translation as described in Fig. 1. After fibrils were removed by centrifugation, the supernatant was transferred to a fresh Eppendorf tube containing a second aliquot of myofibrils. These myofibrils were also incubated for 1 h before being collected by centrifugation. This was then repeated a third and final time. Myofibrillar pellets after the first, second, and third incubations (lanes *a–c*), respectively.

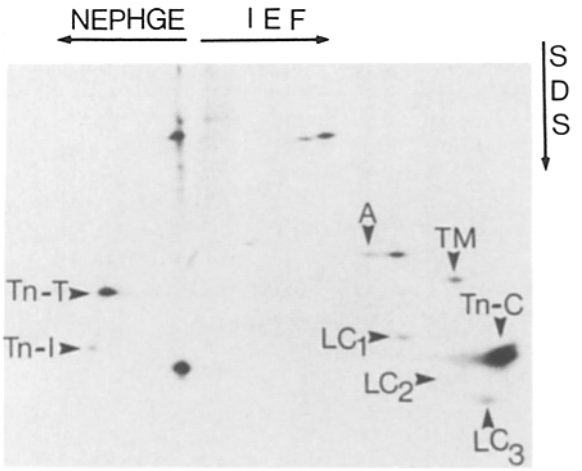


Figure 3. Two-dimensional gel analysis of the labeled proteins incorporated by myofibrils. Myofibrillar pellets after incubation (obtained as in Fig. 1) were analyzed by equilibrium IEF (*right*) and nonequilibrium pH gradient electrophoresis (*NEPHGE*; *left*), followed by SDS-PAGE and fluorography. Each of the designated proteins have been identified by comigration with purified protein standards stained with Coomassie Blue (results not presented). *A*, actin; *LC_1*, myosin LC_1; *LC_2*, myosin LC_2; *LC_3*, myosin LC_3; *TN-C*, troponin-C; *TN-I*, troponin-I; *TN-T*, troponin-T; *TM*, tropomyosin.

resulted from unknown inconstancies in the myofibrillar preparations, since experiments conducted with a single lot of myofibrils but different lysate preparations gave highly consistent results. The reverse experiment was not true [data not shown].) As expected, the incorporation correlated well with the qualitative results shown in Fig. 1 *A*. For individual proteins, the values of incorporation ranged from 3.4 to 27.3% of that synthesized. Incorporation was not proportional to the amount of a protein synthesized. For example, there was less synthesis of the 200- than the 43-kD protein, but substantially more of the 200-kD protein incorporated into the fibrillar pellet. Furthermore, incorporation was unrelated to molecular mass. 14% of both the 32- and the 200-kD polypeptides cosedimented with the myofibrils. Finally, the amount incorporated was not proportional to the relative abundance of a protein in the sarcomere. The labeled band at 20 kD, which is composed predominantly of troponin-C (see Fig. 3), exhibited the highest degree of association with the added myofibrils, yet troponin-C accounts for only 1.3% of myofibrillar mass (Turner et al., 1984; Obinata et al., 1981). Thus, the extent of myofibrillar incorporation for each of the newly synthesized S-proteins appears to be a property unique to each and is not directly related to size, abundance in the sarcomere, or to relative concentration in the reticulocyte lysate. These differences cannot be ascribed to compositional differences in methionine content.

The cosedimentation of labeled S-proteins with the myofibrils increased as one increased the concentration of myofibrils added to the lysate from 0 to 1.0 mg/ml (Fig. 4 *A*). Not all of the protein subunits associated at the same myofibril concentration. For example, the band of 43 kD was barely detectable at 0.2 mg/ml of myofibrils, but was quite prominent at 1 mg/ml. In contrast, the group of protein subunits at ~20 kD comprised the major labeled component(s) which cosedimented with myofibrils at 0.2 mg/ml. The increased association of S-proteins with the pellets was mirrored by a corresponding loss of the same components from the supernatant fraction. This was especially evident for the protein group near 20 kD at 1.0 mg/ml added myofibrils (see Fig. 1 *A*).

TCA-precipitable radioactivity was also measured in the pellet as a function of increasing myofibril concentration (Fig. 5 *A*). A linear relationship was observed between 0.2 and 1.0 mg/ml which extrapolated to zero in the absence of added myofibrils. Approximately 11% of total translated protein cosedimented with the myofibrils at 4°C.

The time course of the incorporation reaction is presented in Fig. 5 *B*. Of the acid-precipitable counts which cosedimented with the myofibrils, 90% did so within 30 min and the reaction appeared to be approaching a plateau by 60 min. The $t_{1/2}$ was <15 min.

The temperature dependence of the reaction is presented in Figs. 5 *C* and 6. Total association of newly synthesized protein with the myofibrils was relatively independent of temperature; only 1.4 times as much incorporation occurred at 37°C than at 4°C and most of that increase took place between 4° and 10°C. Although total incorporation was relatively temperature independent, there were differences between the individual S-proteins (Fig. 6). For example, the proteins of 43, 38, 25, and 18 kD exhibited a significantly higher incorporation at 37°C than at 4°C, whereas the proteins of 95 and 35 kD showed a fall-off in incorporation at elevated temperatures. Incorporation of the remaining proteins appeared to be unaffected by temperature (Fig. 6). Quantitation of this experiment by scanning densitometry is shown in Table II. The ratio of total protein incorporated into the myofibrils at 37°C to that at 4°C was 1.2, virtually identical to that measured independently by TCA precipitation followed by scintillation spectrometry.

Table I. Quantitative Analysis of Myofibrillar Protein Incorporation In Vitro

M_r	Protein subunit	Amount synthesized	Amount incorporated	Percentage incorporated
kD		(10^{-12} g)	(10^{-12} g)	%
200	MHC*	4.8 ± 0.72	0.74 ± 0.22	14.9 ± 4.0
140	C-protein‡	22.0 ± 4.64	2.64 ± 0.74	11.5 ± 1.0
94	α-Actinin§	12.4 ± 3.05	0.75 ± 0.22	6.2 ± 0.8
86	86-kD protein‖	32.5 ± 9.85	1.60 ± 0.42	6.2 ± 1.4
43	Actin¶	22.2 ± 3.39	0.77 ± 0.12	4.0 ± 0.7
38	Tropomyosin**	6.0 ± 1.17	0.14 ± 0.02	3.4 ± 1.0
35	Tn-T (Emb.)‡‡	24.8 ± 6.30	2.64 ± 0.42	14.3 ± 2.8
25	LC1 + Tn-I‡‡§§	9.5 ± 1.13	0.42 ± 0.06	4.8 ± 0.8
20	Tn-C + LC2‡‡§§	7.8 ± 1.22	1.83 ± 0.28	27.3 ± 6.3
18	LC3§§	15.0 ± 4.66	0.38 ± 0.06	6.9 ± 2.4

As described in the text, the amount of radiolabel in each band was determined by scintillation spectroscopy and the amounts synthesized and incorporated were calculated. The quantities represent the amounts synthesized or incorporated per 25-μl sample. Each value is the mean of six experiments ± SEM. M_r, relative mobility of the protein band identified by coelectrophoresis with myofibrillar standards.
* Lowey and Cohen, 1962.
‡ Offer et al., 1973.
§ Ebashi and Ebashi, 1965.
‖ Bahler et al., 1985.
¶ Fornwald et al., 1982.
** Hodges and Smillie, 1970.
‡‡ Greaser and Gergely, 1973.
§§ Weeds and Lowey, 1971.

Tissue Specificity of Protein Associations

To examine the tissue specificity of this in vitro reaction we translated poly(A)$^+$RNA isolated from brain tissue of 19-d-old chick embryos and compared the myofibril incorporation of proteins encoded by this mRNA with those encoded by embryonic muscle mRNA (Fig. 4 *B*). Poly(A)$^+$RNA concentrations were adjusted to achieve equal protein synthesis in the muscle- and brain-directed lysates. Lane *a* of Fig. 4 *B* should be compared with lane *d* of Fig. 4 *A*; these are equivalent experiments except for the different mRNA preparations. Virtually no radioactive proteins could be detected in the myofibril pellet of the brain mRNA experiment in these (15-h) exposures. After a longer (60-h) exposure (Fig. 4 *B*, lane *d*) several radioactive brain proteins were detected in the pellet. The most prominent of these bands migrated at 43 kD and is suspected to be β-actin. Although these results suggest the interesting possibility that actin isoforms are differentially incorporated, it should be noted that at least 10 times more actin was synthesized in the muscle- than the brain-directed lysate reactions. At the level of sensitivity used for

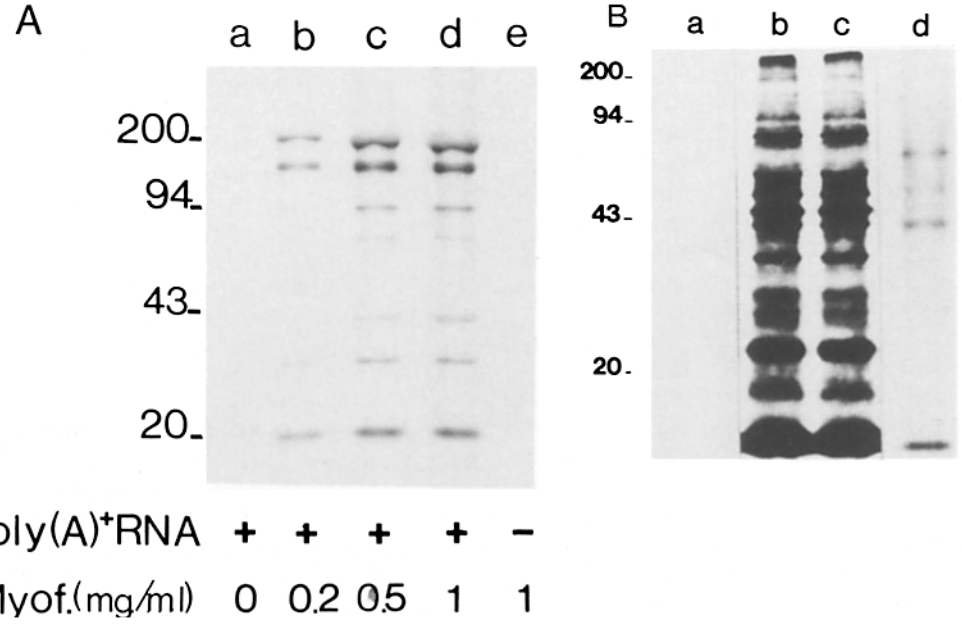


Figure 4. Concentration dependence and tissue specificity. (*A*) The incorporation of ^{35}S-labeled muscle proteins as a function of increasing myofibril concentration (see Fig. 1). Pellet fractions of the translation products incubated with myofibrils at 0, 0.2, 0.5, and 1 mg/ml (lanes *a*–*d*, respectively). Lane *e* contains the myofibrillar pellet of the control sample in which the translation reaction was performed without addition of poly(A)$^+$RNA but then incubated with myofibrils (1 mg/ml) in a manner identical to the other samples. (*B*) Autoradiogram of the translation products encoded by brain mRNA which cosediment with the skeletal muscle myofibrils. Except for the addition of poly(A)$^+$RNA from 19-d-old embryonic chick brain, the procedures were identical to Fig. 1. Myofibril concentration was 1 mg/ml. The brain mRNA concentration was adjusted to give equivalent TCA-precipitable counts to that of the muscle mRNA. All film exposures except lane *d* were for 15 h. Lane *a*, myofibril pellet; lane *b*, supernatant fraction after centrifugation of the myofibrils; lane *c*, total translation products; lane *d*, 60-h exposure of the sample shown in lane *a*.

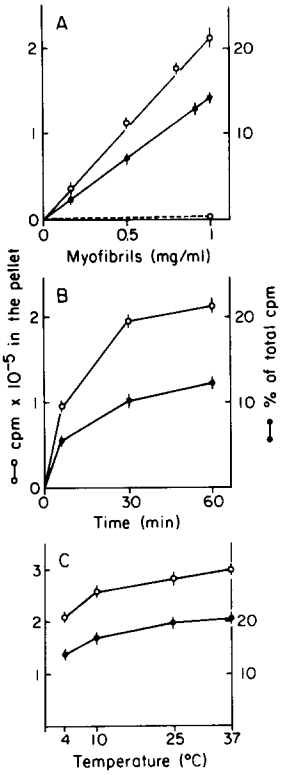


Figure 5. Concentration, time, and temperature dependence of total protein incorporation. Experimental protocol as in Fig. 1 except that (*A*) myofibril concentrations were varied during the 1-h posttranslational incubation on ice, (*B*) the time of posttranslational incubation with myofibrils was varied, or (*C*) the temperature of the posttranslational incubation was varied. The graphs indicate the TCA-precipitable radioactivity recovered in the myofibril pellet. (-o-) Total cpm in myofibril pellet. (-●-) Cpm in the myofibril pellet expressed as a percentage of total protein synthesis. (- -o- -) Total cpm in the myofibril pellet of a brain mRNA-directed translation reaction (*A*).

these experiments, no other reproducible associations were observed.

Filament Specificity of Protein Associations

To further characterize the specificity of this reaction, we prepared filaments from DEAE-purified chicken pectoralis myosin. These filaments were added posttranslationally, in place of the myofibrils, at increasing protein concentrations (20 μg/ml–1 mg/ml). After a standard 1-h incubation on ice, samples were centrifuged for 15 min at 100,000 *g* in an Airfuge to sediment the filaments. A major band of 200 kD, which comigrated with myosin heavy chain, sedimented with the filaments (Fig. 7). Some high molecular mass material did sediment in the absence of any added myosin filaments (lane *a*) but this material did not migrate with the myosin heavy chain on gradient gels (results not shown). The radioactive bands at 25 and 45 kD seen in lanes *e* and *f* of Figs. 7 and 8 represent nonspecific covalent incorporation of methionine into a peptidyl-tRNA and an unknown lysate protein, respectively (discussed in Jackson and Hunt, 1983). Neither of these background bands cosedimented with added synthetic filaments (lane *a*). In similar experiments, the myosin light chains and C-protein have also been found to cosediment with these filaments (Goldfine, S. M., and D. A. Fischman, unpublished observations). Actin did not appear to bind to the myosin filaments, suggesting that the MgATP levels in the lysate were sufficient to inhibit the formation of actomyosin. The presence of a creatine kinase/creatine phosphate–driven ATP-generating system in the lysate mixture could account for this result.

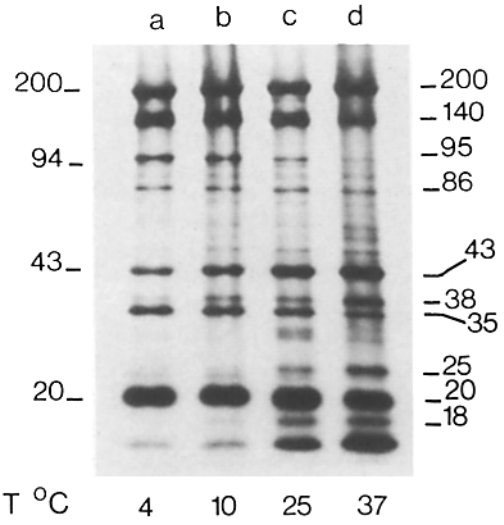


Figure 6. Selectivity of protein incorporation as a function of temperature. Experimental protocol as in Fig. 1 except that temperature was varied (as indicated) in the posttranslational incubations with myofibrils.

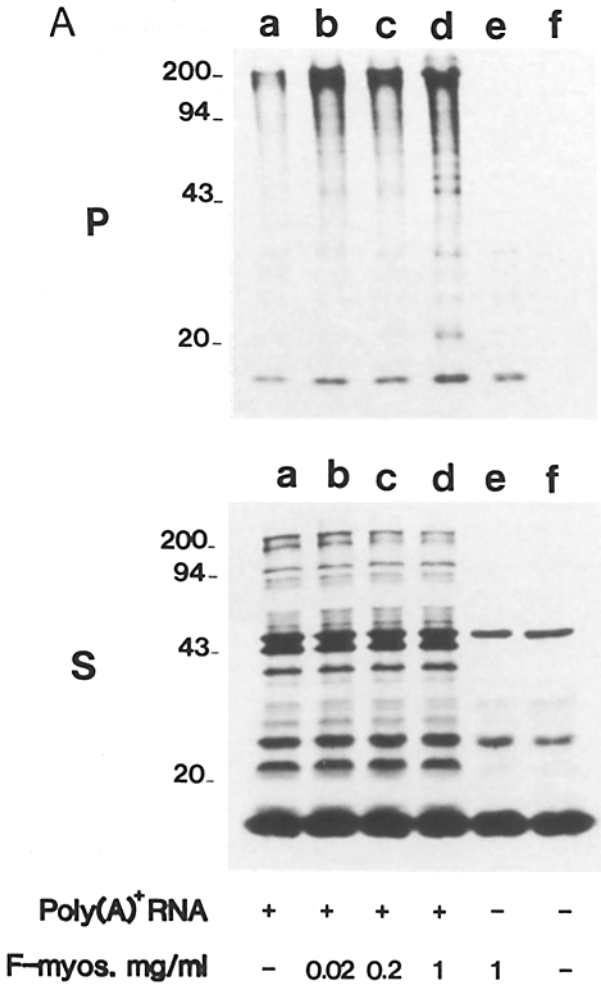
Table II. Quantitation, by SDS-PAGE Densitometry, of the S-Proteins Recovered in the Final Pellet at 4 and 37°C

M_r	Protein subunit*	4°C* D_p	4°C* %T	37°C* D_p	37°C* %T	D_p37°C/D_p4°C*
kD						
200	MHC	1,120	17	1,250	14	1.12
140	C-protein	1,190	18	970	11	0.82
95	α-Actinin	480	7	50	1	0.10
86	86-kD protein	180	3	160	2	0.89
43	Actin	290	5	1,100	12	3.79
38	Tropomyosin	80	1	670	7	8.78
35	Tn-T (Emb.)	630	9	390	4	0.62
25	LC1 + Tn-I	70	1	420	4	6.00
20	Tn-C + LC2	1,670	25	1,340	15	0.80
18	LC3	130	2	390	4	3.00
Total incorporation of S-proteins		5,840		6,740		1.15

Lanes *a* and *d* of Fig. 6 were analyzed by densitometric scanning. The integrated film density for each of the protein bands in the myofibrillar pellet (D_p) at 4 and 37°C are indicated. *%T*, percentage of synthesis of each S-protein relative to total radioactivity in the myofibrillar pellets after an incorporation at each of these two temperatures.

Myosin filaments treated with the zero-length cross-linker 1-ethyl-3-3-dimethylamino propyl carbodiimide were also used in this assay. These filaments, which did not dissociate upon dialysis against high salt buffer, incorporated significantly less material from the lysate than unfixed filaments (Fig. 7 *B*, lanes *a* and *c*). Scintillation counting of the filament pellets revealed that the cross-linked filaments incorporated <10% of the TCA-precipitable radioactivity of the control filaments.

Finally, the association of newly synthesized polypeptides with synthetic actin filaments was examined (Fig. 8). Rabbit F-actin was added at increasing concentrations (10 μg/ml–0.7 mg/ml) under conditions identical to those used for the myosin filament experiments. A band at 43 kD was found to cosediment with the added F-actin in a concentration-dependent manner. Although less prominent, a band at 95 kD was



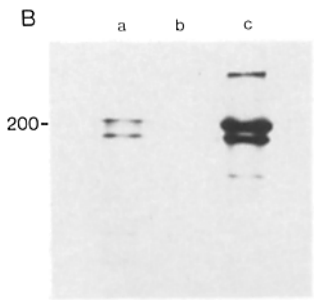


Figure 7. Association of newly synthesized proteins with synthetic (reconstituted) myosin filaments. (*A*) Synthetic thick filaments, prepared from column-purified myosin, were added to posttranslational lysates at the indicated final protein concentrations. After 1 h of incubation at 4°C, the samples were centrifuged at 12,000 *g* for 3 min and the pellets discarded. Negligible TCA-precipitable radioactivity was recovered in this pellet. The supernatants were recentrifuged in an Airfuge at 100,000 *g* for 15 min. The final pellets were dissolved in 9.5 M urea and aliquots corresponding to 1/10 of the pellet samples and 1/50 of the supernatants were displayed by 10% SDS-PAGE. The top fluorogram (*P*) displays the labeled protein cosedimenting with the synthetic filaments; the bottom (*S*) contains the labeled proteins remaining in the supernatant fraction. The bands of 25 and 45 kD seen in lanes *e* and *f* of the supernatant fraction are artifactual and are present with or without the addition of mRNA (Jackson and Hunt, 1983). (*B*) Association of newly synthesized proteins with cross-linked synthetic myosin filaments. Synthetic myosin filaments were cross-linked with 1-ethyl-3-3-dimethylamino propyl carbodiimide as described in the text, before being incubated in the lysate at a concentration of 1 mg/ml. Fluorogram of the upper portion of a 5–18% gradient gel is shown. Lane *a*, those polypeptides that cosedimented with the cross-linked filaments. Lane *b*, those polypeptides that sedimented with the addition of buffer alone. Lane *c*, those polypeptides that cosedimented with filaments which were not cross-linked.

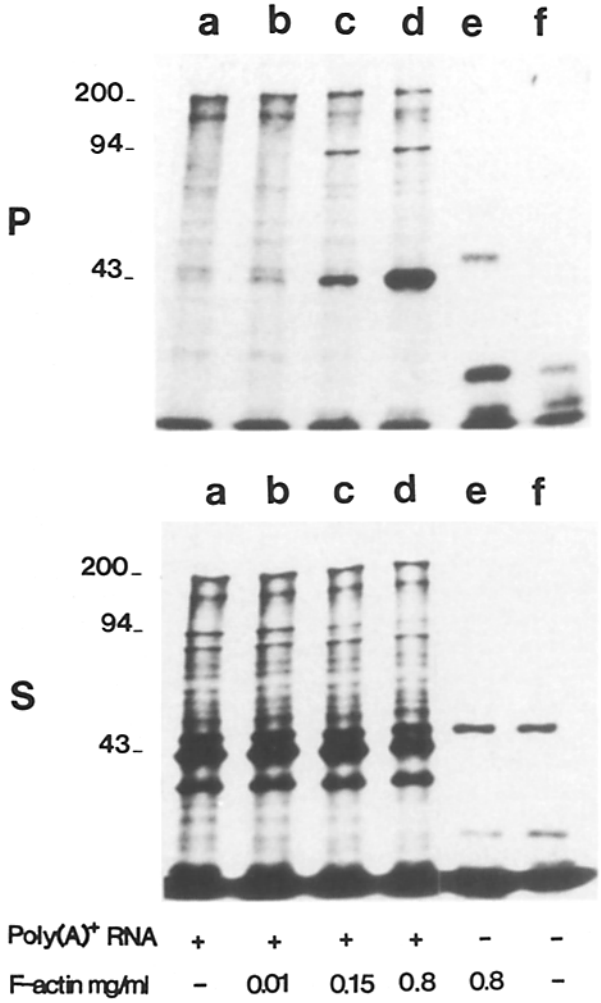


Figure 8. Association of newly synthesized proteins with synthetic F-actin. Rabbit F-actin was added to the posttranslational supernatants at the indicated final protein concentrations. Remaining procedures as in Fig. 7. The labeled material of 25 and 45 kD which cosedimented with F-actin (lanes *e* and *f*) was released from the pellet with one additional wash (data not shown).

also detected in the pellet at actin concentrations >0.15 mg/ml. These proteins coelectrophoresed with α-actin and α-actinin, respectively. Several other high molecular mass proteins were observed in the pellet but their recovery was unrelated to the addition of F-actin (Fig. 8, lane *a*). Unlike filamentous myosin, F-actin did capture the 25- and 45-kD background polypeptides (lanes *a* and *f*). These polypeptides were released from the F-actin pellet with an additional wash, whereas the 43- and 95-kD polypeptides were not.

Discussion

We have described a cell-free system in which only the S-proteins, of all the muscle proteins synthesized in vitro, exhibited posttranslational association with adult myofibrils. The mechanisms underlying this association are not under-

stood. Several arguments can be raised in support of a specific incorporation reaction in contrast to nonspecific protein adhesion. First, the associated labeled proteins did not readily elute from the myofibrils, even after five successive washes in myofibril buffer. Second, if the nonionic detergent (Triton X-100) or the competing protein (BSA) were omitted from the incorporation reaction, many non-myofibrillar proteins did cosediment with the myofibrils. Third, cross-linked myofilaments bound very little newly synthesized protein compared to control myofilaments. Fourth, this incorporation was found to be tissue specific, in that very few of the brain-encoded proteins cosedimented with the myofibrils and the most prominent of those proteins which did associate was a molecule which coelectrophoresed with actin. Fifth, incorporation was filament specific: only the thick filament proteins, myosin and C-protein, cosedimented with exogenously added pure filaments, and only two proteins of 43 and 95 kD cosedimented with added F-actin. The latter two proteins were probably actin and α-actinin, respectively, both of which would be expected to associate with F-actin. Tropomyosin, which also might have been expected to bind to added F-actin, was not incorporated, at least under these experimental conditions. It should be noted that tropomyosin exhibited significant temperature-dependent incorporation with whole myofibrils (see Fig. 6), and the experiments reported with F-actin (Fig. 8) were all performed at 4°C which minimized tropomyosin incorporation. This observation is consistent with the results of Drabikowski and Gergely (1962), who found that the extractability of tropomyosin from actin was highly temperature sensitive.

Present evidence does not permit us to distingiush end addition to preexisting filaments from intra-filament exchange; in fact, both reactions might occur concurrently. The cross-linking experiments are compatible with an exchange reaction, but cross-linking of the filaments might also inhibit end addition. Morphological studies will be necessary to distinguish between peripheral and core labeling of the sarcomeres and to clarify whether protein addition occurs at the ends or all along the myofilaments.

One puzzling observation was that only ~10% of the newly synthesized sarcomeric proteins were incorporated into the myofibrils, even though the incorporation appeared to be approaching saturation by 60 min of incubation (Fig. 5 *b*). The concentration of myofibrils (1 mg/ml) was five to six orders of magnitude greater than the amount of each protein synthesized in the lysate. Experiments with message-selected, actin mRNA (Peng, I., and D. A. Fischman, unpublished observations) reveal that >90% of the actin synthesized in the cell-free system is assembly competent. Experiments are in progress to test the assembly properties of the other newly synthesized sarcomeric proteins. Assuming that the bulk of these proteins are also assembly competent, then other possibilities must be considered for the plateau in the incorporation reaction. First, there might be limiting concentrations of a factor(s) required for protein incorporation. Such a factor(s) might be labile and degraded over the course of the 1-h incorporation reaction. Second, the reaction may be saturating a limiting number of binding and/or exchange sites in the myofibrils. An argument against the first and in favor of the second hypothesis was the observation that addition of fresh myofibrils to the reaction supernatant, after sedimentation of the original myofibrils, resulted in additional capture of labeled sarcomeric protein. If this model system reflects the in vivo situation, one must then consider a pathway, probably absent from the reticulocyte lysate, that continually generates this limiting number of incorporation or exchange sites in the sarcomeres.

The observations reported above are consistent with a growing body of literature demonstrating the dynamic exchange properties of myofibrils and myofilaments in striated, smooth, and nonmuscle tissues. These conclusions derive from the following: (*a*) microinjection of fluorescently labeled analogues of the contractile proteins into living cells; (*b*) incubation of isolated myofibrils with fluorescently labeled proteins; and (*c*) examination of polymerization and exchange reactions with the purified proteins.

Microinjection studies (Kreis and Birchmeier, 1980; Kreis et al., 1982; McKenna et al., 1985*a*, *b*) have demonstrated that fluorescently labeled α-actinin and actin are rapidly incorporated into the appropriate regions of the sarcomere in myofibrils of cultured cardiac and skeletal muscle cells. Fluorescent photobleaching experiments, done on these same cell types (McKenna et al., 1985*b*), strongly suggest the rapid exchange of both of these proteins in vivo. Furthermore, microinjected nonmuscle actin isoforms were found to insert into muscle I-bands, and muscle isoforms entered the microfilament bundles of fibroblasts. These data suggest that there is little isoform specificity in the actin exchange reactions. As noted above, we also observed a small amount of brain actin incorporated into the myofibrils in our cell-free system. Gunning et al. (1984) have also shown that when fibroblasts are transfected with expression vectors encoding cardiac α-actin, the muscle-specific isoform is incorporated into the Triton-insoluble, nonmuscle cytoskeleton, presumably as a component of the microfilaments. One caveat should be considered: regional cytoplasmic segregation of the actin isoforms might reflect quantitative and not qualitative differences between the isoforms in their incorporation or exchange reactions. None of the published experiments have ruled out such a possibility.

Although microinjection analyses with myosin are technically more difficult than with actin, tropomyosin, or α-actinin recent studies demonstrate that fluorescently labeled light chain analogues (Mittal et al., 1987) or whole myosin (McKenna et al., 1988) will rapidly incorporate into the A-band of myofibrils or myotubes. Thus, the available in vivo studies suggest that the sarcomeric proteins, myosin, actin, and α-actinin are all in a kinetic equilibrium with a monomer pool of uncertain size in the cytoplasm. Furthermore, there appears to be little isoform specificity in the exchange process.

Fluorescently labeled α-actinin has been shown to bind to isolated myofibrils (Sanger et al., 1984*a*) and to stain the appropriate regions of sarcomeres within detergent-permeabilized cardiac myocytes in culture (Sanger et al., 1984*b*). In contrast to the results of Glacy (1983) and McKenna et al. (1985*a*), fluorescently labeled actin, when added to glycerinated myofibrils, did not stain whole I-bands (Sanger et al., 1984*a*); retention of exogenous actin was limited to regions of the A-band lacking thick and thin filament overlap. Sanger et al. (1984*a*) have postulated an active process of actin incorporation in the living cells which is absent from the glycerinated myofibrils or detergent-permeabilized cell models. It should be noted that fluorescence energy transfer

studies indicate that <5% of actin subunits within actin filaments are exchangeable under physiological salt conditions (Wang and Taylor, 1981; Pardee et al., 1982). Presumably, I-band myofilaments are capped at both ends (Ishiwata and Fumatsu, 1985; Casella et al., 1987), thus reducing monomer addition or removal from these sites. Our data are in agreement with these prior observations. Only 4% of the newly synthesized actin was captured by the myofibrils, suggesting that this molecule is not readily exchangeable under the conditions used. The capture of newly synthesized actin in the form of actomyosin is unlikely in this assay because there is excess Mg^{++} and an ATP-generating system in the lysate.

Our observations on the association of newly synthesized myosin heavy chain with both synthetic myofilaments and myofibrils are consistent with a number of biophysical studies on myosin exchange. For example, Saad et al. (1986*a*, *b*), have recently applied the technique of fluorescence energy transfer to measure myosin assembly and exchange in vitro. In confirmation of earlier sedimentation velocity (Josephs and Harrington, 1966) and pressure jump studies (Davis, 1981), the fluorescence energy transfer experiments suggest that thick filament assembly occurs by a condensation polymerization reaction in which a critical concentration of 40 nM unpolymerized myosin exists in equilibrium with the synthetic myosin filaments. Furthermore, myosin within synthetic filaments is in rapid exchange ($t_{½}$ <15 min) with this monomer pool. Parallel studies by Trybus and Lowey (1987), with smooth muscle myosin, have extended the concept of a dynamic equilibrium model to that tissue. It remains to be proven that these observations are valid in vivo but they point to the possibility that myosin exchange within striated muscle could be a process of substantial magnitude, even if the rates of such exchange within and between myofibers were reduced substantially by thick filament accessory proteins, such as C-protein (Offer, 1972; Offer et al., 1973; Reinach et al., 1983), M-protein (Masaki and Takaiti, 1972; Trinick and Lowey, 1977), or myomesin (Grove et al., 1984). We are currently investigating the effects of these accessory proteins on the capture of myosin by synthetic filaments in the lysate system.

Although many questions remain unanswered about the posttranslational incorporation reactions, the experimental system reported above provides a new and novel approach to the problem of myofibrillogenesis. By varying the source of input mRNAs or the composition of the "capture" substrate, it may be possible to identify and quantitate subtle differences in the associative properties of the various contractile protein isoforms. This can easily be done by using homogenous mRNA preparations generated with recombinant transcription systems or by message selection with cDNA probes. While not the focus of this report, it has not escaped our notice that this system should have direct applicability to cytoskeletal systems in nonmuscle cells. For example, will stress fibers from fibroblasts incorporate sarcomeric proteins?

Finally, all of the experiments reported here have analyzed posttranslational incorporation reactions. Cotranslational studies require myofibrillar preparations totally free of RNase activity and yet sufficiently native for the assembly and exchange reactions. Experiments of this type are in progress.

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