NONUNIFORM RATES OF TURNOVER OF MYOFIBRILLAR PROTEINS IN RAT DIAPHRAGM

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INTRODUCTION

In recent years much information has been gained on the structure of the contractile proteins in muscle and of the myofibril. Relatively little, however, has been learned concerning the assembly and turnover of this organelle in living striated muscle. The present studies were undertaken to determine whether in the adult animal the myofibril turns over as a unit or whether the individual myofibrillar proteins are synthesized and degraded at different rates. In embryonic muscle, there is strong evidence that the various contractile proteins are synthesized at distinct rates (1) on separate polysomes (2, 3) and that they even first appear at distinct times during embryogenesis (3, 4). Previous studies of the turnover of the myofibril in adult muscle have been inconclusive. Two groups have reported that heavy meromyosin, light meromyosin, actin, and tropomyosin are synthesized and degraded at different rates in vivo (5, 6). Others, however, have suggested that the myofibril has a definite life-span (7, 8).

We have used the rat diaphragm maintained in vitro and measured the rate of incorporation of labeled acids into actin, tropomyosin, and the heavy and light chains of myosin. The present studies indicate nonuniform rates of synthesis of the contractile proteins, and thus suggest heterogeneous rates of degradation.

MATERIALS AND METHODS

Normal and hypophysectomized rats (60–80 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) Hypophysectomized rats were used 2–3 wk after removal of the pituitary; animals which gained weight during this period were discarded. Diaphragms were removed from rats, and each diaphragm with attached ribs was incubated in 4 ml of Krebs-Ringer bicarbonate buffer (9) containing 11 mM glucose, radioactive leucine or lysine, and a complete set of the remaining amino acids (nonradioactive) at the approximate concentrations found in rat plasma (10). The tissues were incubated at 37°C with continuous shaking. At various times, they were removed from the medium, and the diaphragms were cut away from the associated ribs and intercostal muscles. Ten hemidiaphragms were pooled and then transferred into 20 vol of cold potassium phosphate buffer (0.005 M, pH 7.0) containing 0.04 M potassium chloride and 50% glycerol. After incubation overnight at 4°C, the buffer was decanted, and the muscles were stored in fresh glycerol-buffer at −20°C until analysis. Before fractionation of the proteins, the radioactive diaphragms were mixed with a large number of nonradioactive diaphragms, which had been similarly treated with the glycerol-buffer, to give a total of about 10 g of muscles. Diaphragm muscle was used as nonradioactive carrier in order that extraction of labeled proteins and carrier protein would be identical.

The pooled muscles were drained, washed to remove glycerol-buffer, and minced with scissors and razor blades in the cold. One-quarter of the mince was used for preparation of actin, as described by Dowben et al. (11) and Seraydarian et al. (12). Tropomyosin, free of troponin, was extracted from a second quarter of the mince according to the method of Hartshorne and Mueller (13). The rest of the muscle mince was used to isolate myosin. Initially, pyrophosphate extraction and DEAE-cellulose chromatography (14) were performed, and then the

myosin extracts were passed through cellulose phosphate (15). Further purification of myosin was achieved by low ionic strength precipitation and ammonium sulfate fractionation (16). The purified myosin was separated into its heavy and light chain components according to the carbonate-citrate method (17).

Protein concentration was determined routinely in all samples by the method of Lowry et al. (18), using bovine serum albumin as a standard. The Lowry method was standardized by the Kjeldahl method (19), using bovine serum albumin as the standard. The conversion factors (Folin-to-Kjeldahl) were: myosin, 0.90; myosin heavy chains, 0.88; myosin light chains, 0.88; tropomyosin, 0.91; actin, 0.88. These values were the average of two determinations on each of two preparations of protein prepared as described above.

For determinations of radioactivity, aliquots of protein were precipitated with 2 vol of cold 10% trichloroacetic acid (TCA) after 1 mg of bovine serum albumin was added as carrier. The samples were heated at 95°C for 10 min and the precipitates collected and washed with cold 5% TCA on Millipore filters (0.45 μ pore size) (Millipore Corp., Bedford, Mass.). Samples containing [^{14}C]protein were dried and counted in a Nuclear-Chicago Low Background Counter (background, 1–2 cpm) (Nuclear-Chicago Corp., Des Plaines, Ill.). Samples containing [^{3}H]protein were counted by liquid scintillation spectrometry according to Wool and Cavicchi (20).

To determine the amino acid composition of the purified proteins, samples of the proteins (1–2 mg) were hydrolyzed for 22 h at 110°C in 1 ml of hydrochloric acid which had been redistilled under nitrogen. The samples were then dried overnight in a vacuum desiccator, dissolved in buffer, filtered, and analyzed on a Beckman model 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

The validity of these experiments rests upon two crucial points: (*a*) that the extraction procedures yield pure proteins, and (*b*) that relative rates of incorporation of leucine and lysine reflect rates of protein synthesis. The extracted proteins appeared pure on the basis of sedimentation patterns in the ultracentrifuge and electrophoresis on polyacrylamide gels. However, slight contamination (1–2% by weight) of myosin heavy chains with myosin light chains was found. The incorporation of leucine and lysine into protein was studied, because these amino acids had been reported to be present in the various contractile proteins of other species in almost identical amounts (21–23). As shown in Table I, direct analysis of the purified proteins from rat diaphragm confirmed this conclusion. In addition, other experiments in this laboratory (A. L. Goldberg, unpublished) indicate that carbon atoms of leucine and lysine are not converted into other amino acids by skeletal muscle extracts. Furthermore, after incubation of the diaphragms with either [^{3}H]leucine, [1-^{14}C]leucine, or [U-^{14}C]lysine, at least 95% of the label incorporated into protein chromatographed with the original amino acid. No other definite peaks of radioactivity were demonstrable. Thus the relative rates of incorporation of these compounds into the contractile proteins are a measure of the relative synthetic rates. (The untestable assumption of this argument is that the various contractile proteins are synthesized from the same precursor pool.)

As shown in Table II, the diaphragms from normal rats incorporated [^{14}C]- and [^{3}H]leucine into their contractile proteins at varying rates. Differences in the specific activities of the contractile proteins were consistently observed even though the various experiments were carried out under very different conditions (i.e., different amounts of radioactive amino acids were used, different labeled amino acids, different amounts of nonradioactive carrier, and different incubation times). These observations were made at times during which the incorporation of labeled amino acids into total muscle protein proceeded at a linear rate (A. L. Goldberg and S. Martel, unpublished observations).

Of the various contractile protein studied, tropomyosin appeared to be synthesized most rapidly and myosin heavy chains least rapidly. In all the experiments, label was incorporated into the light chains of myosin at approximately twice the rate as into the heavy chains. Actin was also consistently more radioactive than myosin heavy chains, although the magnitude of this difference was variable. Furthermore, since leucine and lysine are present in somewhat lower concentrations in actin than in the heavy chain (Table I), these differences in incorporation probably underestimate the actual differences in rates of synthesis of the two proteins. In all but one experiment, radioactivity in actin was intermediate between that of the two myosin fractions.

DISCUSSION

These results suggest that in adult muscle, as in embryonic tissues (1–4), the myofibril does not

TABLE I

Amino Acid Analysis of Diaphragm Muscle Contractile Proteins

Amino acid	Myosin	Myosin heavy chain	Myosin light chains	Actin	Tropomyosin
Lys	10.56 ± 0.69	9.73 ± 0.45	9.3 ± 0.14	6.76 ± 0.74	13.75 ± 0.68
His	1.06 ± 0.81	1.43 ± 0.53	1.2 ± 0.14	2.14 ± 0.31	Trace
Arg	5.20 ± 0.72	5.43 ± 0.64	3.9 ± 1.13	5.16 ± 0.43	4.75 ± 0.44
Asp	10.37 ± 0.90	9.65 ± 0.38	11.2 ± 0.0	10.12 ± 0.90	10.9 ± 0.44
Thr	5.80 ± 0.77	5.4 ± 0.72	3.9 ± 0.14	6.42 ± 0.88	2.75 ± 0.26
Ser	5.0 ± 0.78	5.0 ± 0.42	4.0 ± 0.14	6.84 ± 0.58	4.0 ± 0.32
Glu	17.43 ± 2.11	17.70 ± 1.71	20.3 ± 0.57	12.46 ± 1.74	26.95 ± 1.35
Pro	2.87 ± 0.96	3.13 ± 1.97	4.50 ± 1.13	1.14 ± 1.80	0.00 ±
Gly	6.70 ± 1.71	5.83 ± 1.31	6.05 ± 0.21	8.04 ± 0.55	1.70 ± 0.20
Ala	8.93 ± 0.26	8.6 ± 0.64	9.55 ± 0.42	9.08 ± 0.42	12.75 ± 0.84
Cys	0.50 ± 0.74	0.43 ± 0.49	Trace	2.42 ± 4.60	Trace
Val	6.0 ± 0.51	5.75 ± 0.76	5.50 ± 0.28	5.42 ± 0.78	2.90 ± 0.60
Met	2.0 ± 0.33	2.10 ± 0.42	2.8 ± 0.0	3.06 ± 1.82	2.25 ± 0.06
Ile	5.30 ± 0.40	5.13 ± 0.20	4.35 ± 0.07	6.92 ± 0.79	3.85 ± 0.06
Leu	9.37 ± 0.75	9.50 ± 0.63	7.75 ± 0.07	8.40 ± 0.89	11.30 ± 0.25
Tyr	1.50 ± 0.90	2.20 ± 0.28	1.55 ± 0.07	2.60 ± 0.623	0.90 ± 0.92
Phe	2.26 ± 0.77	2.75 ± 0.88	4.15 ± 0.21	3.26 ± 0.30	0.25 ± 0.25

Data is expressed as moles of each amino acid per 100 mol of recovered amino acid. Each number represents the average ± the standard error of the mean of at least two determinations performed on each of two separate preparations of the proteins.

turn over as a unit. Evidence has been obtained that incubated rat diaphragms synthesize the various contractile proteins at distinct rates. If the present results are applicable to muscles in the intact animal, they further suggest that myofibrillar proteins are also degraded at different rates. (Otherwise, those proteins synthesized most rapidly would accumulate in the muscle disproportionately.) In addition, nonuniform rates of protein synthesis were also observed in nongrowing muscle from hypophysectomized rats, in which rates of protein synthesis and degradation should be equally balanced. These experiments thus suggest that tropomyosin turns over slightly more rapidly than myosin light chains, which in turn appear significantly more labile than actin or myosin heavy chains. Several distinct polypeptides compose the light chain fraction (24), and it is presently unclear whether these various components are also synthesized at unequal rates.

Our present results appear to contradict the report of Zak et al. (8) that the myofibrillar proteins in the heart appear to turn over at uniform rates. One possible explanation could be that the myofibrils of cardiac and skeletal muscles are assembled differently, although such a conclusion appears unlikely. These workers estimated relative degradation rates in the animal with the double-label approach previously employed in studies of protein degradation in liver (25, 26). A number of problems may exist in the use of such a technique to follow turnover in peripheral tissues. One potential complication would be the continued secretion of radioactive amino acids from the liver to peripheral tissues (10) long after the initial injection of radioactive tracers into the animal. Such an extended exposure would tend to decrease the apparent differences between the half-lives of muscle proteins (29).

Another possible explanation of these apparently contradictory observations would be that the present results hold only for tissues incubated in vitro. During incubation in vitro, both skeletal muscle (R. M. Fulks and A. L. Goldberg, in preparation) and cardiac muscle (27, 28) generally undergo net protein degradation, which only occurs in the organism during such conditions as starvation or muscle atrophy (30). Possibly the in vitro incubation also alters the pattern of protein synthesis, such that the various contractile proteins are produced nonuniformly. However, evidence supporting this possibility is lacking; in fact, our preliminary experiments also suggest nonuniform rates of leucine incorporation into contractile

TABLE II

Incorporation of Amino Acids into Myofibrillar Proteins of Rat Diaphragm (cpm/mg)

Experiment	Myosin (total)	Myosin heavy chains	Myosin light chains	Actin	Tropomyosin
Normal rats					
(1) [^{3}H-4,5-1]leucine 0.52 μCi/ml					
6 h	87	78	111	92	154
(2) [U-^{14}C]leucine 0.2 μCi/ml					
3 h	31	27	57	29	62
5 h	42	32	67	41	85
Hypophysectomized rats					
(3) [U-^{14}C]leucine 0.1 μCi/ml					
6 h	13	9	22	15	22
(4) [U-^{14}C]leucine 0.25 μCi/ml					
5 h	69	51	95	93	105
	122	90	155	116	126
(5) [U-^{14}C]lysine 0.52 μCi/ml					
4 h	39	30	72	61	78

In each experiment, at least five diaphragms from normal and hypophysectomized rats were incubated with the radioactive amino acid for the times indicated. The final concentration of leucine and lysine in these experiments was 0.1 mM. The radioactive samples were counted for a sufficiently long time to give standard errors ranging between 3 and 5%.

proteins in the intact organism. Since the completion of these studies, a report by Funabiki and Cassens has appeared (1972. *Nat. New Biol.* **236:**249) also suggesting nonuniform rates of turnover of myofibrillar proteins in rabbit skeletal muscle.

The observation that light and heavy chains of myosin appear to be degraded at distinct rates could help account for anomalous findings about myosin turnover in vivo. On the basis of apparent multicomponent decay kinetics, it has been claimed that myosin has a distinct life-span, rather than undergoing exponential decay like other cell proteins. The existence of two distinct components with different half-lives in myosin preparations would predict rather complex decay curves for the labeled proteins. The present observations, however, cannot account for earlier claims that heavy meromyosin has a significantly longer half-life than light meromyosin (5, 6). On the contrary, our results would suggest that the heavy meromyosin fraction, which contains myosin light chains, should appear to turn over more rapidly. McManus and Mueller (31) have previously pointed out a number of technical problems inherent in the earlier investigations.

The existence of distinct degradative rates for the myofibrillar proteins would be in accord with growing evidence that proteins composing other cell organelles (e.g., the mitochondrion, endoplasmic reticulum, or cell membrane [25, 26]) also turnover at heterogenous rates. In mammalian liver, Dehlinger and Schimke (26) have found an inverse correlation between the rates of degradation of cell proteins and their molecular weights. This general correlation, obtained for both soluble and membrane proteins, does not appear true for myofibrillar proteins in the diaphragm. The very large myosin heavy chain (mol wt approximately 200,000) appears quite stable by comparison with the much smaller tropomyosin molecule (mol wt approximately 35,000) or the even smaller myosin light chains (mol wt approximately 20,000) (4).

Myofibrillar proteins are believed to turn over

relatively slowly in the animal, although recent measurements indicate significantly more rapid renewal than described previously (32–34). The rate of turnover of these proteins must influence the rate at which the contractile properties of a muscle can change in response to hormone activity (35), neural influences (36), etc. For example, it is well documented that denervation or cross-innervation can alter the rate of contraction and the rates of myosin ATPase (36). Generally, it has been assumed that these effects involve the complete assembly of a new myofibrillar system. In light of the present findings, such altered properties could occur simply by replacement of certain of the more rapidly turning over proteins (e.g., light chains of myosin) while other components remained unaltered. The manner in which these various contractile proteins join or leave the highly organized sarcomere are completely unknown at present. Such questions are of special interest with regard to the mechanisms of muscle growth and atrophy, during which both the average rates of synthesis and degradation of myofibrillar proteins are altered (30).

SUMMARY

Experiments were undertaken to determine whether in skeletal muscle the various myofibrillar proteins are synthesized and degraded as a unit or whether they turn over separately. Measurements of amino acid incorporation were performed on rat diaphragms incubated in vitro. Actin, tropomyosin, mosyin heavy chains, and myosin light chains from this muscle were found to contain equivalent concentrations of leucine and lysine. Nevertheless, the diaphragms incorporated radioactive leucine and lysine into those proteins at different rates. Thus the various contractile proteins appear to be synthesized at distinct rates. Similar results were obtained in diaphragms of nongrowing (hypophysectomized) rats, suggesting that the proteins are also degraded nonuniformly. The heavy chain of myosin appears to turn over more slowly than actin and appreciably more slowly than tropomyosin or the light chains of myosin.

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