Change in the Reactivity of Myosin during Muscle Contraction*

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SUMMARY

Myosin can be labeled with 1-fluoro-2,4-dinitrobenzene in frog muscle. The incorporation into myosin is decreased during isotonic contraction. In contrast no difference is found in the incorporation into the sarcoplasmic proteins of contracting and resting muscle. Myosin also reacts with iodoacetate in frog muscle. However, this reaction does not depend on the functional state of the muscle.

The reactivity of myosin in contracting muscle follows a similar pattern in model systems. The dinitrophenyl content of myosin isolated from myofibrils dinitrophenylated in the relaxed state is higher than that of myosin from myofibrils dinitrophenylated while contracting. Incorporation of 1-fluoro-2,4-dinitrobenzene into pure frog myosin is greatly increased in the presence of additions which relax myosin as compared to the additions which contract the fibrils. With the same additions, frog actin, or myosin-free myofibrilar residues show no change in the dinitrophenylation. Furthermore, the reactivity of contracting and relaxed myofibrils remains the same when 1-fluoro-2,4-dinitrobenzene is substituted by iodoacetate.

These data show that 1-fluoro-2,4-dinitrobenzene is a valuable probe to differentiate the functional states of myosin in myofibrils and living muscle.

Recently we have shown an ATP-dependent reaction of rabbit skeletal myosin with 1-fluoro-2,4-dinitrobenzene (1). Free ATP markedly increased the rate of dinitrophenylation of this myosin whereas MgATP2- or actin inhibited it. Myosins from different types of muscles and from various sources also exhibited the ATP-dependent dinitrophenylation reaction (2). Furthermore, the ATP effect on the dinitrophenylation was rather specific for myosin since it was not apparent in several enzymes that utilize the L4TP effect on the dinitrophenylation was rather specific for myosin since it was not apparent in several enzymes that utilize ATP (2).

It is known from the work of Cain and Davies (3) and Cain, Infante, and Davies (4), that FDNB readily penetrates into frog muscle fibers. We have shown that in addition to the soluble enzymes, myosin also reacts with FDNB in frog muscle (5). We wish to report in this paper that the reactivity of myosin with FDNB changes during muscle contraction. To elucidate the effect of FDNB on myosin in the intact muscle we have determined the reaction of FDNB with myosin in contracting and relaxed frog myofibrils and actomyosin. A preliminary report of this work has appeared (6).

EXPERIMENTAL PROCEDURE

Myofibrils—The procedure of Ulbrecht and Ulbrecht (7) was followed to prepare myofibrils from frog muscle (Rana pipiens). The various functional states of myofibrils were established in 100 mM Tris-HCl buffer (pH 7.0) and 0.13 ionic strength. In addition to Tris-HCl the medium contained, for relaxation: 3.0 mM ATP, 1.0 mM EGTA, and 10 mM KCl (8); for contraction: 3.0 mM ATP, 3.0 mM MgCl2, 0.1 mM CaCl2, and 21 mM KCl (8); and for rigor: 80 mM KCl. In a few experiments relaxation was produced by 1.0 mM ATP, 1.0 mM MgCl2, 1.0 mM EGTA, and 19 mM KCl (9), and contraction was produced by 1.0 mM ATP, 1.0 mM MgCl2, 0.1 mM CaCl2, and 27 mM KCl (9). These functional states of the fibrils were controlled (9) by ATPase activity determinations (0.02 to 0.05 μmole of Pi per min per mg of relaxed fibrils and 0.42 to 0.55 μmole of Pi per min per mg of contracting fibrils), and by syneresis (0.30 to 0.35 ml of precipitate for relaxed fibrils and 0.08 to 0.10 ml of precipitate for contracting fibrils). Syneresis was also measured in the presence of 25 mM potassium phosphate buffer (pH 7.0) instead of the 100 mM Tris-HCl buffer. No difference was found between these buffers.

The incorporation of 3H-FDNB or 3H-iodoacetate into myofibrils was determined at 25°C, unless otherwise indicated. The medium contained 1.0 mg of fibrils per ml in either 25 mM potassium phosphate buffer (pH 7.0) or 100 mM Tris-HCl buffer (pH 7.0), and all the additions necessary to obtain relaxation, contraction, or rigor, at ionic strength of 0.13. The suspension was stirred with 1H-FDNB for 23 min or with 3H-iodoacetate for 20 min, then 400-fold molar excess of cysteine was added over the reagent, and the mixture was stirred continuously for an additional 5 min. The myofibrils were precipitated with 4% trichloroacetic acid, washed three times with 2% trichloroacetic acid, once with 0.1% trichloroacetic acid, and finally dissolved by homogenization in 2% NH4OH. Samples were taken from this homogenate to determine the protein concentration by the biuret method (10) and the radioactivity by liquid scintillation counting (1).

When the myosin component of the dinitrophenylated fibrils was isolated (Table III), DNP-cysteine was removed from the

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1 The abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; DNP-, 2,4-dinitrophenyl-.
fibrils by several washings with 0.1 M KCl. The fibrils were then extracted with 0.3 M KCl, 0.15 M potassium phosphate buffer, pH 6.6, and 0.01 M ATP at a concentration of 5 mg of fibrils per ml. The mixture was stirred slowly at 0° for 30 min, centrifuged at 35,000 × g, and the supernatant solution was diluted with 15 volumes of cold distilled water. The myosin precipitate was collected, reprecipitated once, and dissolved in 0.6 M KCl. This myosin solution was centrifuged in the Spinco preparative ultracentrifuge at 150,000 × g (average force in the rotor 50) in the presence of 0.01 M ATP, 0.01 M MgSO₄, and 0.02 M Tris-HCl buffer, pH 7.4, at 0° for 2 hours. The upper two-thirds of the supernatant solution was decanted, precipitated with trichloroacetic acid, washed with trichloroacetic acid, and dissolved in NH₄OH to determine the incorporation of ³H-FDNB into the myosin component of the myofibrils.

To prepare “myosin-free” myofibrillar residues the fibrils were extracted five times with the solution containing 0.3 M KCl, 0.15 M phosphate, pH 6.6, and 0.01 M ATP (each extraction for 30 min as described before). The final residue was washed 10 times with 0.04 M KCl, pH 7.0, to remove Pi and ATP. These myofibrillar residues still possessed some ATPase activity, 0.05 μmole of P₁ per min per mg, in the presence of Mg²⁺ or Ca²⁺ at low ionic strength.

Actomyosin and Myosin—These were prepared from frog muscle as described recently (5) except that no cysteine was used in the isolation of myosin.

Actin—The muscle residue obtained after extraction of myosin was washed with 0.4% NaHCO₃, then with distilled water, and finally dried with acetone. To increase the yield of actin, the dried powder was extracted with 0.3 M KI and 1 mm ATP, pH 7.0 (11). The partially polymerized actin solution was dialyzed against large volumes of 0.1 M KCl, pH 7.0, to transform all the actin into the fibrillar form. The F-actin was sedimented at 150,000 × g and the pellet dissolved by homogenization in 0.1 M KCl, pH 7.0.

The incorporation of ³H-FDNB into “myosin-free” myofibrillar residues, actomyosin, myosin, or actin was determined under the same conditions as described above for the incorporation of ³H-FDNB into myofibrils.

Living Muscles—The rectus abdominis, pectoralis major, and cutaneous pectoris muscles from large frogs (R. pipiens) were carefully dissected as one unit and cleaned of adherent tissues. A pair of such muscle units was obtained from the same frog by cutting along the linea alba between the right and left rectus.

This unit was chosen, instead of the usual rectus muscle, in order to isolate enough pure myosin. A previously weighed hook was tied with thread to one end of each muscle unit and a previously weighed plastic block was sewn on the other end. The paired muscles were weighed on an analytical balance and were used only when their weights were equal within 5%. The hooks were then connected to the isometric levers and the plastic blocks were firmly attached with plastic screws to the muscle chambers. Subsequently each muscle unit was oxygenated in 70 ml of normal Ringer’s solution (12) in its own temperature-controlled chamber at 2-4°. (The cooling jackets of both chambers were connected to the Lauda-Brinkmann circulator K-2R.) After 30 to 60 min the response of the paired muscles to electric stimuli was tested; if their contractility was grossly different they were discarded. The muscles were stimulated 12 times per min with a duration of 1 msec under a load of 1.5 g (12). Under these conditions at 3°, the contraction time of the muscles was determined to be 0.86 sec to reach the maximal shortening with 1.79 sec being required to complete a contraction and relaxation cycle. Thus, the stimulated muscles have spent 17% of the total time in shortening and 36% of the total time in the combined shortening and subsequent lengthening phase of the mechanical activity.

Treatment of the muscles with ³H-FDNB was performed as follows. A stock ³H-FDNB in normal Ringer’s solution was prepared at 0° to contain 0.10 to 0.15 mM ³H-FDNB. The Ringer’s solution of each of the paired muscles was exchanged for 70 ml of this 0.10 to 0.15 mM ³H-FDNB solution. Both muscles were treated with the ³H-FDNB-Ringer’s solution at 2-4° for exactly the same time (50 to 60 min) while they were oxygenated. During the FDNB treatment, one muscle was stimulated while the other was resting. In control experiments either both muscles were stimulated or both were resting. The FDNB treatment was stopped by exchanging the FDNB-Ringer’s solution for normal Ringer’s solution of room temperature. After 2 min the normal Ringer’s solution was exchanged for a fresh one. Five such Washes were performed. The part of the muscle which participated in the contraction was cut from the lever and bath attachment in a way that the upper and lower parts of the muscle were discarded. The identical part of the resting muscle was cut. The muscles were wiped with tissue paper and weighed on the analytical balance (the weights ranged from 300 to 550 mg).

Treatment of the muscles with ³H-iodoacetate was carried out under the same conditions as described for the ³H-FDNB treatment with the exception that the ³H-iodoacetate concentration was 0.20 to 0.25 mM.

Determination of Incorporation of ³H-FDNB into Sarcoplasmic Proteins of Living Muscles—The muscles at the end of the ³H-FDNB treatment were chopped with the scissors and extracted with 100 ml of 0.04 M KCl, pH 7.0, with stirring in the cold room for 1 hour. After centrifugation at 27,000 × g, the supernatant fluid was saved, and the residue was blended with 100 ml of 0.04 M KCl, pH 7.0, at 4° for 5 sec. The blendate was centrifuged at 27,000 × g and the supernatant combined with the previous one. The sarcoplasmic proteins were precipitated by 10% trichloroacetic acid, washed with 0.1% trichloroacetic acid, dissolved in NH₄OH, and their radioactivity determined.

Determination of Incorporation of ³H-FDNB into Myosin of Living Muscles—The residue obtained after extraction of the sarcomplasmic proteins was extracted with 30 ml of a solution containing 0.6 M KCl, 0.05 M Tris-HCl (pH 7.4), and 5.0 mM ATP, at 4° for 20 hours. The dissolved actomyosin was freed of connective tissue by centrifugation, then precipitated by dialysis against 0.01 M KCl (pH 7.0) in the cold room. (During the 24 hours of dialysis the 100 volumes of 0.01 M KCl were exchanged eight times.) The actomyosin precipitate was collected and dissolved in 0.6 M KCl. The myosin was isolated from this actomyosin by dissociation with 0.01 M MgATP*, at 1.0 mg of actomyosin per ml, in 0.6 M KCl (pH 7.5) at 0°, and subsequent centrifugation in the rotor 50 of the Spinco for 1 hour (modification of method in Reference 13). The myosin in the supernatant was precipitated with 3% trichloroacetic acid, centrifuged, dissolved in NH₄OH, and its radioactivity determined. The purity of this myosin preparation was controlled in the analytical ultracentrifuge. For these experiments the Spinco supernatant was dialyzed against frequent changes of 0.01 M KCl (pH 7.0), at 0°; the precipitated myosin was centrifuged and dissolved in

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The stock 3H-FDNB solution was prepared and standardized as described recently (1); the specific activity of the 3H-FDNB used in this work was 10 $\mu$Ci per pmole. The labeled iodoacetic acid was recrystallized with nonlabeled iodoacetic acid (1) to obtain a specific activity of 10 $\mu$Ci per pmole. The stock solution was neutralized to pH 7.0. Radioactivity was determined by liquid scintillation counting in a dioxane-based scintillation solvent (1).

RESULTS

Myofibrils and Actomyosin—Table I shows the incorporation of 3H-FDNB into frog myofibrils at 25° when the fibrils are relaxed, contracting, or in rigor. At the four different 3H-FDNB concentrations, the rate of incorporation into the relaxed fibrils is the highest throughout while no significant difference appears between contracting fibrils or those in rigor. Taking the incorporation into the relaxed fibrils as 100%, the dinitrophenylation of the contracting fibrils or those in rigor varies from 62 to 72% and averages 68%. Results similar to those described in Table I were obtained when frog actomyosin was used instead of myofibrils. The pH and ionic strength of these experiments resembles those of Ringer's solution for the study of the incorporation of 3H-FDNB into the proteins of frog muscles (Tables IV and V), although there is a difference in the temperature.

When the temperature of the experiments with myofibrils was lowered to 3°, the temperature of the experiments with the living muscles, no contraction of the fibrils was observed. Thus the sedimenting volume of the fibrils was equal in the presence of either MgATP$^{2-}$ and Ca$^{2+}$, or ATP and EDTA (MgATP$^{2-}$ and ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid), and their ATPase activity was also equal in the presence of these additions. Incorporation of 3H-FDNB into myofibrils at 3° was roughly the same with any of these additions, indicating that a difference in the functional state of the fibrils is required to observe a difference in the incorporation of FDNB.

Myofibrils, relaxed, contracting, or in rigor, showed reactivity identical with 3H-iodoacetate at 25°. In these experiments 125 to 1,000 moles of 3H-iodoacetate were added per 500,000 g of fibrils resulting in incorporation of 0.10 to 0.26 mole of 3H-carboxymethyl groups. Although this incorporation was rather small, the very high specific activity of the 3H-iodoacetate would have allowed the detection of any significant difference. Thus it appears that iodoacetate is not a reagent to probe the functional states of the fibrils.

Components of Myofibrils—We studied the reaction of 3H-FDNB with various components of myofibrils to determine the origin of the specific dinitrophenylation which appears from the data of Table I. The four different 3H-FDNB concentrations used in these experiments were identical with those used for the myofibrils (Table I).

Table II compares the incorporation of 3H-FDNB into frog myosin, actin, and myofibrillar residues in the presence of ATP, EDTA, and KCl (condition for relaxation of fibrils), ATP, MgCl$_2$, CaCl$_2$, and KCl (condition for contraction of fibrils), or KCl alone (condition for rigor of fibrils). In the ease of myosin, the incorporation of 3H-FDNB is about 2 times higher in the presence of ATP, EDTA, and KCl than with KCl alone. The smallest incorporation is found in the presence of ATP, MgCl$_2$, CaCl$_2$, and KCl; this is the result of the protecting effect of MgATP$^{2-}$ on the dinitrophenylation of myosin (1). In contrast to these changes in the rate of dinitrophenylation of frog myosin, dinitrophenylation of frog actin remains the same in the presence of various additions. Myofibrillar residues, obtained after exhaustive extraction of myosin, show a small decrease in the incorporation when the effects of either ATP, MgCl$_2$, CaCl$_2$, and KCl, or KCl alone are compared to those of ATP, EDTA, and KCl. This small change is caused by the remaining myosin...
in the residues as evidenced by their ATPase activity (see "Experimental Procedure"). Therefore, these data indicate that the removal of myosin from the fibrils abolishes the specific changes in their dinitrophenylation.

The data of Table II show clearly that of the components of myofibrils only myosin exhibits the characteristic changes in the dinitrophenylation which are observed with myofibrils (Table I). Direct proof was also obtained to show that the increased incorporation of $^3$H-FDNB into relaxed myofibrils is caused by the increased dinitrophenylation of their myosin component. Table III shows a typical experiment which compares the incorporation of $^3$H-FDNB into myofibrils and into the myosin isolated from the dinitrophenylated fibrils. The highest $^3$H-DNP content is seen in the fibrils dinitrophenylated in the relaxed state and in the myosin isolated from the same fibrils. The $^3$H-DNP content of myofibrils, dinitrophenylated while contracting or in rigor, and that of myosin from the same fibrils is significantly lower. The percentage of incorporation differs between myofibrils and the myosin component of the fibrils. There is 70% incorporation into fibrils contracting or in rigor as compared to relaxed fibrils, and 52% incorporation into the myosin from fibrils contracting or in rigor as compared to myosin from relaxed fibrils. This difference in the incorporation is caused by the fact that only the dinitrophenylation of myosin changes with the functional states of the fibrils, whereas the dinitrophenylation of the other myofibrillar proteins remains constant.

Living Muscles—At 2-4° the muscles were stimulated in Ringer's solution containing 0.10 to 0.15 mm $^3$H-FDNB for 50 to 60 min. No rigor was developed under these conditions. The work performed by the FDNB-treated muscles varied from 400 to 850 g times cm per g of muscle. The work performance of the untreated muscles was in the same range.

The uptake of $^3$H-FDNB by resting muscles was determined in a few experiments. The muscles were treated with Ringer's solution containing 0.15 mm $^3$H FDNB at 3° for 60 min, and subsequently washed with normal Ringer's solution five times. To determine the incorporation, pieces of 20 to 40 mg were cut from the washed muscles, gently drained, transferred into previously weighed vials, and weighed on the analytical balance. Scintillation solvent was added into the vials and the radioactivity was counted. The calculated $^3$H-DNP concentration in the muscle was over 0.30 mm, indicating considerable labeling of muscle proteins. In all these experiments no essential difference was found in the radioactivity between the paired resting muscles.

Table IV compares the incorporation of $^3$H-FDNB into the myosin and sarcoplasmic proteins of resting and contracting muscles. In the four experiments shown, the incorporation into myosin varies from 0.34 to 0.75 mole per 500,000 g of protein. The $^3$H-DNP content of myosin from the contracting muscle is consistently less than of myosin from the resting muscle, on average 83%. The incorporation of $^3$H-FDNB into the sarcoplasmic proteins is higher than into the myosin: 1.54 to 2.16 moles/500,000 g of proteins. However, the $^3$H-DNP content of sarcoplasmic proteins from the contracting muscle is 99% of that of the resting muscle. These data indicate that during contraction only the reactivity of myosin is changed with FDNB but not that of sarcoplasmic proteins. It should be mentioned that about 4 mg of myosin were isolated from 100 mg of muscle, wet weight, in these experiments; this amount represents about 70% of the myosin in these muscles. Thus the difference in the labeling refers to the main bulk of the myosin of contracting and resting muscles.

Table V summarizes our results of the changes in the incorporation of $^3$H-FDNB into myosin and sarcoplasmic proteins of frog muscles of different functional states.

The muscles were treated with $^3$H-FDNB-Ringer's solution as described under "Experimental Procedure." To calculate the percentage of incorporation, incorporation into the resting muscle was taken as 100% in the case of contracting versus resting type of experiment, whereas in the control experiments the higher incorporation was taken as 100%. Results are given with ± standard error of means.
In both cases the incorporation of FDNB into myosin is decreased during contraction. Fifty pairs of muscles were analyzed, when one was contracting and the other resting. Forty pairs of muscles were analyzed as controls when either both muscles were contracting or both were resting. Incorporation of $^3$H-FDNB into the myosin of these muscles varied from 0.22 to 0.87 mole/500,000 g of protein. Incorporation into the myosin from the contracting muscle is 82%, compared to the myosin from the resting muscle. This difference is statistically significant when compared to the controls. In contrast, incorporation of $^3$H-FDNB into the sarcoplasmic proteins of the functionally different muscles is the same. Furthermore, there is no difference between the percentage of incorporation into myosin or into the sarcoplasmic proteins from muscles when twitch versus twitch or resting versus resting are compared. These results extend the data of Table IV establishing the correlation between muscle contraction and decreased incorporation of FDNB into myosin.

The same types of experiments, as shown in Table V, were performed with $^3$H-iodoacetate instead of $^3$H-FDNB. The incorporation of iodoacetate into myosin during contraction did not differ from that into myosin of resting muscles. Taking the incorporation into myosin of resting muscle as 100%, that into myosin of contracting muscle was 97% with a standard error of ±6.0. Similarly, no difference was found in the incorporation of $^3$H-iodoacetate into the sarcoplasmic proteins of working and resting muscles.

**DISCUSSION**

A simple method is described to differentiate the functional states of myosin in myofibrils and living muscle. This is based on a selective reactivity of FDNB with myosin in contracting and resting muscles. The specificity of the FDNB reaction is indicated by the following: (a) there is no specific reaction when contraction of myofibrils is inhibited by lowering the temperature, (b) the specificity holds true only for myosin but not for the sarcoplasmic proteins of the working muscle, and (c) FDNB cannot be substituted by iodoacetate either in the fibrils or in the intact muscles. We have shown previously that FDNB reacts predominantly with the globular head section of the myosin molecule, and that this reaction is markedly influenced by ATP, actin, or both (1). Thus a functional change in myosin can be detected by FDNB. Naturally, effects as shown by FDNB may be reproduced by other reagents which react with the globular part of myosin. Indeed, our preliminary findings revealed this effect with iodoacetamide or N-ethylmaleimide.

The results of this paper show clearly that the phenomenon which occurs in isolated contractile proteins of frog muscle also occurs in living and active muscle. Thus the reaction of FDNB with myosin in living muscle is in qualitative agreement with this reaction in myofibrils (cf. Tables I and III with Tables IV and V). In both cases the incorporation of FDNB into myosin is decreased during contraction.

The decreased incorporation of FDNB into myosin during muscle contraction may not result from a decreased permeation. A marked increase in permeability was observed in electrically stimulated frog muscles (14). Furthermore, any change in the permeability would have been reflected in a uniform decrease of incorporation into all proteins, i.e., myosin and soluble proteins. However, the data of Tables IV and V show a selective decrease for myosin.

It is reasonable to assume that the decreased incorporation of FDNB into myosin is the result of a decreased reactivity of amino acid residues of myosin in the working muscle. This may be the consequence of the combination of actin with myosin during contraction, because it has been shown that actin protects cysteine and tyrosine residues of myosin from reaction with FDNB (1). This assumption is supported by the identical decrease of incorporation of FDNB into contracting myofibrils and in rigor (Table I), as it is generally assumed that actin and myosin are maximally combined in the state of rigor. Another explanation for the decreased reactivity of FDNB is a conformational change of myosin in the contracting muscle, since our recent work indicated an ATP-induced conformational change in myosin (2). Therefore, the possibility exists that either ATP, or actin, or both, change the conformation of myosin during contraction. This would support the hypothesis that the sliding filament mechanism of contraction may be operated by conformational changes of the myosin molecule (15, 16).

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