Studies on Myosin from Red and White Skeletal Muscles of the Rabbit

I. ADENOSINE TRIPHOSPHATASE ACTIVITY *

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SUMMARY

Myosin obtained from red skeletal muscle of rabbit has a lower adenosine triphosphatase activity than myosin from white skeletal muscle. Structural differences between the two types of myosin are suggested by the higher apparent activation energy of the calcium-activated adenosine triphosphatase reaction catalyzed by red muscle myosin, by the higher rate of inactivation at pH 7.5 to 9.5, and by the previously reported slower rate of tryptic digestion of myosin from red muscle. No evidence has been found for the presence in red muscle, or in myosin prepared from red muscle, of inhibitors or inactivators of myosin adenosine triphosphatase.

Two types of myosin also differ with respect to the pattern of activation of adenosine triphosphatase by sulfhydryl reagents. At low ionic strength myosin from red muscles is activated by N-ethylmaleimide and myosin from white muscles is unaffected, whereas at high ionic strength both myosins are activated. Four moles of p-chloromercuribenzoate per 10^6 g of myosin are needed for maximal activation of fresh myosin from red muscles, but only 2 moles are required per 10^6 g of myosin from white muscles. This difference disappears in a few days, suggesting a change in the availability of sulfhydryl groups.

We have also reported that the rate of tryptic digestion of myosin-R^2 is slower than that of myosin-W (3). Differences in tryptic digestion of red and white muscle myosin from pigeon also were reported (5). The work to be presented in this paper deals with some aspects of both types of myosin in greater detail.

EXPERIMENTAL PROCEDURE

The following rabbit muscles were used for the preparation of myosin: soleus, semitendinosus, erector, and intertransversarius (red muscles); vastus lateralis and adductor magnus (white muscles). Myosin was prepared by the dilution technique essentially as described by Perry (6). Preparations were also made by the method of Luchi, Kritcher, and Cohn (7) and by the method of Szent-Györgyi (8).

ATPase activities were determined by measuring the liberation of inorganic phosphate according to the method of Fiske and SubbaRow (9). Protein was determined by the biuret reaction.

RESULTS

Although our myosin-R preparations appear homogeneous in the ultracentrifuge, a finding analogous to that reported by Barany et al. (4), the question arises whether the lower ATPase activity might be due to an inhibitor or inactivator of myosin ATPase present in red muscle. We used several approaches to clarify this problem. First, the linear dependence of activity on enzyme concentration suggests the absence from myosin-R of either an inhibitor or an inactivator (Fig. 1). Second, when myosin-R and myosin-W were mixed, the activities were additive, which would not be expected if an inhibitor or inactivator were present in myosin-R. Third, we tried to determine whether there might be present in red muscle itself but not in the purified myosin-R a substance that inactivates myosin during preparation. Myosin-W was mixed with a 0.1 mM KCl extract of red muscle from which myofibrils had been removed by centrifugation, or with a 0.3 mM KCl-0.15 M PO_4, pH 6.5, extract. This extract had been dialyzed against 0.04 mM KCl, myosin had been removed, and the extract had been concentrated 4-fold. These mixtures were stored overnight at 0°C, and myosin-W was re-purified by two reprecipitations. The specific ATPase activity of the myosin-W after these treatments was not less than 80%
of the original value. Also, this myosin-W repurified from the red extracts was unaffected by incubation at pH 9.0 for periods up to 10 min under conditions where the inactivation of myosin-R was essentially complete.

Finally, myosin-R and myosin-W prepared by three different methods were compared. Although the absolute values of the specific activities differed depending on the method used—the differences are now being studied in our laboratory—the ratio of myosin-W to myosin-R activities remained essentially the same (Table I). The constancy of the ratio is particularly clear with EDTA-activated ATPase. The variation in the activity ratios of calcium-activated ATPase might result from differences in residual magnesium (10–12).

In view of the differences between the ATPase rates and the rate of tryptic digestion of myosin-W and myosin-R (3), we investigated the effect of varying the KCl concentration. According to a recent report by Warren, Stowring, and Morales (13), KCl may act by disrupting some structural features of myosin. A comparison of the effect of KCl on ATPase rates for myosin-W and myosin-R with and without Ca++ is shown in Fig. 2. Qualitatively, the effect of KCl is the same on both types of myosin, i.e. the rates increase monotonically with KCl concentration in the absence of added Ca++, while they decrease monotonically in the presence of 10 mM CaCl₂. Calcium markedly activates ATPase activity at low KCl concentrations, but inhibits at high KCl concentrations (Fig. 2A). Calcium becomes inhibitory to myosin-R at lower KCl concentration than in the case of myosin-W. Notwithstanding these differences, however, it seems that the effect of KCl on both myosin-W and myosin-R is essentially the same. This is brought out by plotting the activities as percentages of the maximal activity, in which case the myosin-R and myosin-W curves become superimposable (Fig. 2B).

### Table I

<table>
<thead>
<tr>
<th>Method of preparation of myosin</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cation-activated ATPase</td>
<td>EDTA-activated ATPase</td>
</tr>
<tr>
<td>Myosin-W</td>
<td>0.67</td>
</tr>
<tr>
<td>Myosin-R</td>
<td>0.22</td>
</tr>
<tr>
<td>Myosin-W</td>
<td>1.17</td>
</tr>
<tr>
<td>Myosin-R</td>
<td>0.49</td>
</tr>
<tr>
<td>LiCl- (NH₄)SO₄ (7)</td>
<td></td>
</tr>
<tr>
<td>KCl (8)</td>
<td></td>
</tr>
</tbody>
</table>

We also studied the effect of temperature on the ATPase activities of both myosin-W and myosin-R. In the presence of 1 mM EDTA and 0.5 mM KCl, no difference in temperature dependence was observed (Fig. 3). An apparent enthalpy of activation (ΔH*) at 25°C for the over-all reaction of 16.4 kcal per mole was observed. In contrast, in the presence of 10 mM CaCl₂, myosin-R was more temperature-dependent than myosin-W (Fig. 4); apparent enthalpies of activation at 25°C being 7.4 and 4.5 kcal per mole, respectively. These values in the presence of calcium were not affected by changes of the KCl concentration between 0.3 and 0.025 M.

We have reported that the dependence of myosin-R ATPase activity on pH does not differ significantly from that of myosin-W (3). However, Barany et al. (4) reported lack of activation of myosin-R ATPase activity at alkaline pH. This apparent contradiction appears to be resolved by our studies at alkaline pH.
FIG. 3. Temperature dependence of EDTA-activated ATPase of myosin-R and myosin-W. The conditions of ATPase assay were, 1 mM EDTA, 0.5 M KCl, 5 mM ATP, 0.05 mM Tris, pH 7.5, 0.2 mg of myosin per ml. O, myosin-W; •, myosin-R. Apparent enthalpy of activation was calculated from the formula $\Delta H^\circ = -R [d \ln V/d (1/T)] - RT$, where $V$ = ATPase activity, $R$ = molar gas constant, and $T$ = absolute temperature.

FIG. 4. Temperature dependence of calcium-activated ATPase of myosin-R and myosin-W. The conditions of ATPase assay were, 10 mM CaCl$_2$, 0.025 M KCl, 5 mM ATP, 0.05 M Tris, pH 7.5, 0.2 mg of myosin per ml. O, myosin-W; •, myosin-R. In which the order of adding the components of the enzyme assay was varied. If myosin was added before ATP, no increase in activity was observed in the alkaline range, in agreement with the findings of Barany et al. (4). Preincubation of myosin-R at pH 9.0 in the absence of ATP led to a rapid loss of ATPase activity, which was counteracted by the presence of ATP (Fig. 5). The ATPase activity of myosin-W was practically unaffected by exposure to pH 9 in the absence of ATP.

FIG. 5. Effect of incubation at pH 9.0 on ATPase activity of myosin-R and myosin-W. In one series of experiments (O, •), myosin (0.2 mg per ml) was added to a reaction mixture consisting of 1 mM EDTA, 0.5 M KCl, 0.05 mM Tris, pH 9.0, at 25°C. At times indicated on the abscissa 5 mM ATP was added, and after 5 min the ATPase reaction was stopped by the addition of trichloroacetic acid (5%). In a second group of experiments ( ), myosin was added to the above reaction mixture, which already contained 5 mM ATP. Additional ATP (1 mM) was added at 5 and 10 min to replace that which was hydrolyzed by myosin. Samples were taken for Pi analysis at the times indicated on the abscissa and 5 min later. Inorganic phosphate was determined after addition of trichloroacetic acid (5%). The values shown in the figure correspond to the differences between these pairs of measurements. Values for zero incubation at pH 9.0 were determined by adding myosin to the assay system containing ATP and terminating the reaction with trichloroacetic acid after 5 min. The specific activity for myosin-R not incubated at pH 9.0 was 0.63 pmole per mg per min, that for myosin-W 1.97 pmole per mg per min. O, myosin-W; •, myosin-R; •, myosin-R + 5 mM ATP.

**TABLE II**

Loss of ATPase activity of myosin-R at alkaline pH values

<table>
<thead>
<tr>
<th>Buffer*</th>
<th>pH of incubation</th>
<th>Activity lost in 2 min</th>
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</thead>
<tbody>
<tr>
<td>Tris</td>
<td>7.5</td>
<td>15</td>
</tr>
<tr>
<td>Tris</td>
<td>8.5</td>
<td>28</td>
</tr>
<tr>
<td>Tris + glycine</td>
<td>9.0</td>
<td>49</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.5</td>
<td>54</td>
</tr>
</tbody>
</table>

* All buffers were 0.04 M.
Table II summarizes the results of experiments in which myosin-R was preincubated without ATP for 2 min at various pH values.

Differences in the structure of the enzymatically active region of myosin-W and myosin-R are suggested by differences in the change of ATPase activity when thiol reagents are added. The effect of p-chloromercuribenzoate is shown in Fig. 6. We have reported earlier that about twice as many moles of p-chloromercuribenzoate per 10^5 g of protein must be added to myosin-R as must be added to myosin-W in order to maximally activate the ATPase activity (1). After more extensive investigation, it appears that this difference is apparent only with freshly prepared myosin (assayed within 48 hours after killing the rabbits) (Fig. 6). Aging of the myosin solution leads to a shift in the optimal amount of p-chloromercuribenzoate to 2 moles per 10^5 g of myosin-R, the value characteristic of myosin-W. Concomitant with this shift, the absolute value of the ATPase activity of both myosin-W and myosin-R decreases.

Modification of myosin with N-ethylmaleimide also brings out a difference between myosin-W and myosin-R. When tested at low ionic strength (KCl = 0.1 M), the activity of myosin-W hardly changes, while that of myosin-R is increased, often to the activity of myosin-W (Fig. 7). At high ionic strength (0.55 M KCl), both myosin-R and myosin-W are activated by N-ethylmaleimide. In contrast to the findings with p-chloromercuribenzoate, the age of the preparation did not affect the concentration of N-ethylmaleimide required for activation or the differences between myosin-R and myosin-W at low ionic strength.

**DISCUSSION**

The data presented in this paper extend and amplify previous reports (1-4) on differences between myosin from white and red skeletal muscles of rabbit.

The low ATPase activity of myosin-R, in comparison with that of myosin-W, in the presence of Ca++, EDTA, or actin + Mg++ (3) suggests, but does not prove, a difference in the structure of the two proteins. A priori, the lower ATPase activity of myosin-R could be due to the presence of inactive proteins or of inhibitors, or to partial inactivation of myosin-R during the preparation.

The presence of inhibitors, as distinct from inactive impurities, in the myosin-R preparations is unlikely in view of the additivity of activities of myosin-W and myosin-R in mixtures of the two enzymes, and the proportionality of activity of myosin-R to enzyme concentration. The presence of inactivating substances in the red muscle itself is unlikely, since extracts of red muscle did not modify the ATPase activity of myosin-W. It is particularly noteworthy that myosin-W to which a red muscle extract was added or mixtures of myosin-R and myosin-W show no change in stability at alkaline pH.

The presence of substantial amounts of impurities is unlikely in view of the ultra centrifugal homogeneity of myosin-R, as reported by Barany et al. (4) and also found by us.

A number of other observations reported in this paper also tend to exclude the possibility that the lower ATPase activity of myosin-R is due to the presence of impurities that would, as it were, dilute the active enzyme. The ratio of the activities of myosin-W and myosin-R varies depending on whether K+, Ca++, EDTA, or actin + Mg++ (3) are the activators. The apparent enthalpy of activation (ΔH*) of the calcium-activated myosin...
ATPase is quite different for the two preparations, the values being 4.5 and 7.4 kcal per mole for myosin-W and myosin-R, respectively. The Arrhenius plots are linear for both preparations, in agreement with earlier reports (14–18, cf. Reference 19), indicating that the conformation of the enzyme-ATP complex does not change within the range of 0–35°.

Values of ΔH* from the literature for calcium-activated myosin ATPase, measured under widely varying conditions, including pH, ionic strength, and Ca++ concentration, vary from 2.4 to 12.9 kcal per mole (14–17, 19). Thus, a comparison with our values is difficult. However, our results obtained under identical conditions clearly show that myosin-W has a lower apparent activation energy than myosin-R.

A striking difference between myosin-W and myosin-R is the lability of the latter at alkaline pH. In view of the protection by ATP against alkaline inactivation, these findings explain the earlier reported lack of activation of myosin-R ATPase at alkaline pH (4), since myosin was added to the reaction mixture before ATP.

These observations, together with the earlier reported slower rate of tryptic digestion of rabbit myosin-R (3) suggest a structural difference between myosin-R and myosin-W. It is still premature to conclude that there has to be a difference in the primary structure at the active site, since structural changes in regions remote from the enzymatic site might lead to altered activity. It is interesting that the ATPase activities of myosin-W and myosin-R showed the same dependence on KC1 concentration when expressed as a percentage of the maximal activity, suggesting that any structural changes produced by increasing the KC1 concentration (13) are the same for myosin-R and myosin-W.

The experiments dealing with the reactivity of sulfhydryl groups in myosin-R and myosin-W also show a difference between the two preparations. The fact that with freshly prepared myosin-R—but not with aged myosin-R—more p-chloromercuribenzoate is needed for activation than with myosin-W suggests that some of the sulfhydryl groups of myosin-R (not involved in enzymatic activity) may be more readily available to p-chloromercuribenzoate. The disappearance of the difference in a few days may reflect a structural change in myosin-R leading to the masking of these groups, since the number of sulfhydryl groups in myosin-W and myosin-R is the same (4) and no change takes place in their number during this time period.

The reduction or disappearance of the difference between the specific ATPase activities of myosin-R and myosin-W after N-ethylmaleimide treatment suggests the possibility that modification by N-ethylmaleimide might reduce or eliminate some structural differences between myosin-R and myosin-W. The lack of an activating effect of N-ethylmaleimide on myosin-W at low ionic strength is not due to a slower reaction rate, since no activation is observed at higher N-ethylmaleimide concentrations (cf. Fig. 7) or on exposure for longer periods of time. The suggestion by Warren, Stowring, and Morales (13) that mercurial activators of ATPase act by preventing the inactivation of myosin by high salt concentrations may require some revision, since ATPase activities in the presence of N-ethylmaleimide or p-chloromercuribenzoate in high concentrations of KC1 exceed those observed at low KC1 concentrations in the absence of thiol reagents. The ATPase activity of heavy meromyosin also shows activation by phenylmercuric acetate with added KC1 over and above the rate without added KC1 (20). Furthermore, the marked differences between the effects of N-ethylmaleimide and p-chloromercuribenzoate may require the postulation of a more complex mechanism of activation.

The present results suggest that myosin exists in two different molecular forms in rabbit skeletal muscle, the form having the higher maximal velocity of ATP hydrolysis predominating in the rapidly contracting white muscles, and the other predominating in the more slowly contracting red muscles.

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REFERENCES

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