Actin-Activated Adenosine Triphosphatase Activity of Native and N-Ethylmaleimide-Modified Cardiac Myosin from Normal and Thyrotoxic Rabbits

SURATH K. BANERJEE AND EUGENE MORKIN

SUMMARY The Ca\(^{2+}\)-ATPase activity of cardiac myosin is increased in thyrotoxic animals. However, the physiological significance of this observation is uncertain since, in living muscle, Mg-ATP is hydrolyzed by myosin under the stimulating influence of actin. In this study, we have compared the actin-activated ATPase activity of myosin from euthyroid (myosin-N) and thyrotoxic (myosin-T) rabbits and the derivatives of myosin-N and myosin-T formed by blocking the most rapidly reacting class of thiols (SH\(_{\text{N}}\)) with N-ethylmaleimide (NEM). Also, we have studied the activity of these myosins in the presence of a complex of troponin and tropomyosin that confers calcium sensitivity on the system. \(V_{\text{max}}\) for the actin-activated ATPase of myosin-T was about 168\% greater than for myosin-N. The apparent dissociation constant for actin, \(K_{\text{app}}\), for myosin-T was about 42\% of the normal value. After NEM modification, \(V_{\text{max}}\) and \(K_{\text{app}}\) for NEM-modified myosin-T and myosin-N decreased, becoming essentially the same for both myosins. In the presence of troponin-tropomyosin complex, the actin-activated ATPase of myosin-T exhibited calcium sensitivity that could be reduced by thiol modification. These results suggest that the SH, thiols or the region near these groups are important to the actin-activated ATPase of myosin-N and are essential to the increased activity of myosin-T. Also, they suggest that the changes in the enzymatic properties of myosin induced by thyroxine may be responsible for altering the contractile properties of the heart.

Methods

EXPERIMENTS ON RABBITS

Thyrotoxicosis was induced in young male albino rabbits weighing 1.0-1.5 kg by daily intramuscular injections of L-thyroxine (20 \(\mu\)g/100 g body weight) for 14 days. Rabbits were weighed daily, and the dose of thyroxine was reduced to one-half, or was omitted, if the body weight fell to less than 80\% of the initial value. Uninjected littermates were used as controls.

PREPARATION OF MYOSIN

Cardiac myosin was isolated by a modification of the method of Katz et al. as described previously. A final centrifugation was carried out at 125,000 \(g\) for 90 minutes in the presence of Mg-ATP to remove any traces of actin. Saturated (NH\(_4\))\(_2\)SO\(_4\) was added to the supernatant fluid, and the fraction that precipitated between 34\% and 45\% saturation was collected by low speed centrifugation. All procedures were performed at 4°C and all solutions contained 1 mM EDTA.

PREPARATION OF ACTIN AND TN-TM COMPLEX

Actin and TN-TM complex were prepared from the back and leg muscles of euthyroid adult rabbits of the same strain. Actin was extracted from acetone powder at 0°C and purified by gel filtration on Sephadex G-200 according to the method described by Rees and Young. Fractions from the G-actin peak were polymerized in 0.1 M KCl, 1 mM MgCl\(_2\), 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, and 1 mM Tris-Cl (pH 7.6), and stored as F-actin pellets at -20°C. Before each assay, the F-actin pellets were dispersed by gentle homogenization in 10 mM Tris-Cl buffer, pH 7.6, containing 0.1 mM ATP. After removal...
of free nucleotide by addition of 0.05 volume of Dowex 1-X8 in the chloride form, actin was repolymerized by making the solution 0.1 M and 1 mM with respect to KCl and MgCl₂, respectively. TN-TM complex was prepared from purified myofibrils by the method described by Hartshorne and Mueller.

NEM MODIFICATION OF MYOSIN

Myosin that had been dialyzed against 0.5 M KCl-0.05 mM Tris-Cl at pH 7.0 was reacted in an ice bath with an 8-fold excess, on a molar basis, of NEM for 20 minutes. The Ca²⁺-ATPase activity of preparations of NEM-modified myosin-N were 225-282% of the unreacted value; preparations of NEM-modified myosin-T were 88-105% of the unreacted value. NEM-myosins contained about 2.0-2.2 mol of NEM per mol.

ATPase ASSAYS

The actin-activated ATPase of myosin was assayed essentially as described by Eisenberg and Moos in medium containing 50 mM KCI, 0.1 mM CaCl₂, 15 mM Tris-Cl (pH 7.5), 1.5 mM MgCl₂, and 1 mM ATP in a final volume of 4 ml at 37°C. In preliminary experiments it was determined that, under these conditions, the liberation of inorganic phosphate (Pᵢ) was linear for at least 4 minutes. Subsequently, a 2-minute reaction time was routinely used for this assay. The myosin concentration was 0.25 mg/ml, and the actin concentration was varied from 0.14 to 2.0 mg/ml. The Mg²⁺-ATPase of myosin was assayed under the same conditions, except that actin was omitted and the reaction time was 4 minutes. For determination of the actin-activated ATPase in the presence of TN-TM complex, the protein concentrations were as follows: myosin, 0.25 mg/ml; actin, 0.9 mg/ml; and TN-TM complex, 1.0 mg/ml. The calcium sensitivity of the ATPase activity was determined by a comparison of assays obtained with either 0.1 mM CaCl₂ or 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N⁴⁴-tetraacetic acid (EGTA) in the medium. The protein concentrations were determined by measuring $A_{280}$ corrected for light scattering, using an extinction coefficient of $E_{280}^m = 5.6$ for myosin, 11.49 for F-actin, and 3.8 for TN-TM complex.

The assays were terminated by the addition of 2 ml of 10% trichloroacetic acid, and $P_i$ liberated in the reaction was measured by the method of Fiske and Subbarow.

Results

KINETICS OF STEADY STATE ACTIN-ACTIVATED ATPase

Figure 1 shows a double-reciprocal plot of the actin-activated ATPase vs. the actin concentration for cardiac myosin from normal and thyrotoxic rabbits. Under the conditions used for these assays, straight lines were obtained in both cases. The intercepts of these lines give two parameters by which the actin activation kinetics can be characterized. The reciprocal of the ordinate-intercept is the extrapolated maximal steady state rate of hydrolysis at infinite actin concentration, $V_{max}$. The reciprocal of the abscissa intercept gives a parameter, with the dimensions of actin concentration, that can be taken to represent the apparent dissociation constant for actin, $K_{app}$. This parameter may be operationally defined as the actin concentration required to reach half-maximal activation. Also, the inverse of this function is a measure of the apparent affinity of myosin for actin as an activator.

Table 1 shows the kinetic constants $V_{max}$ and $K_{app}$.

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Material</th>
<th>$Mg^{2+}$-ATPase (μmol Pᵢ/mg per min)</th>
<th>$V_{max}$ (μmol Pᵢ/mg per min)</th>
<th>$K_{app}$ (10⁻⁴ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Myosin</td>
<td>0.04</td>
<td>0.73 ± 0.06</td>
<td>37.2 ± 4.9</td>
</tr>
<tr>
<td>Thyrotoxic</td>
<td>Myosin</td>
<td>0.12</td>
<td>1.23 ± 0.05</td>
<td>15.8 ± 1.5</td>
</tr>
<tr>
<td>Normal</td>
<td>NEM-myosin</td>
<td>0.12</td>
<td>0.46 ± 0.04</td>
<td>8.2 ± 1.8</td>
</tr>
<tr>
<td>Thyrotoxic</td>
<td>NEM-myosin</td>
<td>0.24</td>
<td>0.45 ± 0.03</td>
<td>6.1 ± 1.4</td>
</tr>
</tbody>
</table>

Assays were performed as described in Methods. $Mg^{2+}$-ATPase activity has been subtracted from the values shown for actin-activated ATPase activity. Kinetic constants were obtained as described by Cleland, using a weighted least squares fit of the Lineweaver-Burk equation for the data shown in Figures 1 and 2. Values are means ± se. $P_i$ = inorganic phosphate.
obtained from the data shown in Figure 1 by means of a weighted least squares program. \( V_{\text{max}} \) for myosin-T was determined to be 1.23 \( \mu \text{mol Pi/mg per minute} \), which was about 168% greater than normal. Also, these data bring out a difference between these myosins in their apparent affinity for actin. The \( K_{\text{app}} \) for myosin-N was about 37 \( \times 10^{-6} \) M, whereas the \( K_{\text{app}} \) for myosin-T was 16 \( \times 10^{-6} \) M, or about 42% of the normal value. In other words, a lower actin concentration was required to activate the ATPase activity of myosin-T.

Table 1 also shows the rate of hydrolysis of Mg-ATP by myosin-N and myosin-T in the absence of actin. Thus the Mg\(^{2+}\)-ATPase activity of myosin-T was 300% greater than the Mg\(^{2+}\)-ATPase of myosin-N. This intrinsic Mg\(^{2+}\)-ATPase activity was subtracted from the values shown for the actin-activated ATPase.

Figure 2 shows a double-reciprocal plot of the actin-activated ATPase vs. the actin concentration for SH\(_2\)-blocked myosin from normal and thyrotoxic rabbits. The values obtained with the two types of myosin were essentially the same. The kinetic parameters \( V_{\text{max}} \) and \( K_{\text{app}} \) that were obtained from these data are given in the lower part of Table 1. Incorporation of about 2 mol of NEM per mol of myosin-N decreased \( V_{\text{max}} \) to about 63% of the unmodified value. Incorporation of a similar amount of NEM into myosin-T caused the activity to fall even more markedly, to about 37% of the unmodified value. Thus the actin-activated ATPase activities of myosin-N and myosin-T were about the same after NEM modification. Blocking the SH\(_2\) thiols also markedly reduced the \( K_{\text{app}} \) for both types of myosin. The effect on myosin-T was less, so that the \( K_{\text{app}} \) values for NEM-modified myosin-N and NEM-modified myosin-T were indistinguishable.

The Mg\(^{2+}\)-ATPase activity of the SH\(_2\)-blocked myosins also is shown in Table 1. The activity of NEM-modified myosin-N was about 300% greater than the native protein, whereas the activity of NEM-modified myosin-T was about 200% greater.

**EFFECTS OF TN-TM COMPLEX ON ACTIN-ACTIVATED ATPase**

The actin-activated ATPase activity of cardiac myosin from normal and thyrotoxic rabbits also was assayed in the presence of TN-TM complex (Table 2). This activity was markedly inhibited in both types of myosin by adding EGTA and TN-TM complex to the assay medium. When CaCl\(_2\) was added to the system, the actin-activated ATPase of myosin-N and myosin-T became 350% and 395% greater, respectively, than when EGTA was in the medium. Thus, regulation of the actin-activated ATPase activity of myosin-T by calcium and the TN-TM complex appeared to be normal.

The effects of NEM modification of myosin-N and myosin-T are shown in the lower part of Table 2. When SH\(_2\)-blocked myosin-N and myosin-T were assayed in the presence of TN-TM complex, the calcium sensitivity of the system was diminished to about one-half that observed with the unmodified myosins. With CaCl\(_2\) in the medium, the activities of NEM-modified myosin-N and myosin-T were 150% and 200% greater, respectively, than with EGTA. These observations suggest that the SH\(_2\) thiol groups, or possibly the conformation in the region near these groups, are important to the actin-activated ATPase activity of myosin-N and are essential to the enhanced activity of myosin-T.

**Discussion**

The present results indicate that the \( V_{\text{max}} \) for the actin-activated ATPase of cardiac myosin from thyrotoxic rabbits was about 168% greater than normal. The apparent dissociation constant (\( K_{\text{app}} \)) for actin from myosin-T was reduced to about 42% of the normal value, indicating an increase in the affinity for actin as an activator. Blocking the SH\(_2\) thiol groups in myosin-N slightly decreased the \( V_{\text{max}} \) for actin-activated ATPase activity. After modifica-

### Table 2

**Effects of Troponin-Tropomyosin (TN-TM) Complex on the Actin-Activated ATPase of Native and N-Ethylmaleimide (NEM)-Modified Myosin from Normal and Thyrotoxic Rabbits**

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Material</th>
<th>Without TN-TM complex</th>
<th>With TN-TM complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Plus EGTA</td>
</tr>
<tr>
<td>Normal</td>
<td>Myosin</td>
<td>0.28 ± 0.02</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Thyrotoxic</td>
<td>Myosin</td>
<td>0.31 ± 0.04</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Normal</td>
<td>NEM-myosin</td>
<td>0.42 ± 0.05</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>Thyrotoxic</td>
<td>NEM-myosin</td>
<td>0.48 ± 0.05</td>
<td>0.27 ± 0.02</td>
</tr>
</tbody>
</table>

Assays were performed as described in Methods. Values are means ± SD for three experiments.
tion of these groups in myosin-T, the activity was reduced more markedly, so that the values for $V_{\text{max}}$ of the SH$_2$-blocking myosins were about the same. Also, experiments were performed on the actin-activated ATPase activity of myosin-N and myosin-T in the presence of TN-TM complex. In the absence of EGTA, this complex of regulatory proteins inhibited the activity of myosin-T to the level of myosin-N. The addition of CaCl$_2$ to the medium markedly increased the activity of both types of myosin, the activity of myosin-T becoming greater than myosin-N. The calcium sensitivity of these systems was markedly reduced by modification of the SH$_2$ thiols. These results deserve further discussion in terms of the molecular basis for the effects of thyroxine on myosin and the possible physiological significance of these findings.

The enzymatic role of myosin in the contraction process is to catalyze the hydrolysis of Mg-ATP. Actin greatly increases this activity, possibly by accelerating the release of the products of hydrolysis, ADP, and $P_i$, from the myosin heads. Thus it is important to determine whether or not thyroxine administration affects the Mg$^{2+}$-ATPase activity of cardiac myosin in the presence of actin.

Previous studies in thyrotoxic animals have dealt mostly with the activity of myosin when calcium or monovalent cations were used as activators. Beginning with Thyrum and co-workers, it has been appreciated that the Ca$^{2+}$-ATPase activity of cardiac myosin is increased in thyrotoxicosis. The K$^+$(EDTA)- and NH$_4^+$-ATPase activities have been found to be normal. The latter findings probably can be explained by the fact that the mechanism of ATP hydrolysis by myosin is dependent upon whether divalent or monovalent cations are present. In the presence of calcium or magnesium, hydrolysis of ATP proceeds via formation of a series of reaction intermediates that do not occur in the presence of potassium and a divalent metal cation chelator such as EDTA. Thus it seems likely that thyroid hormone administration affects only the reaction pathway(s) involving divalent metal cations.

Studies on the Mg$^{2+}$-ATPase activity of actomyosin from thyrotoxic animals have yielded conflicting results. The activity of myofibrils from guinea pig hearts has been reported to be elevated, whereas the activity of natural cardiac actomyosin from rabbits was found to be normal. The reason for this discrepancy is uncertain, but it may be related to the difficulty of preparing myofibrils and natural actomyosin from heart with consistent purity and activity. In any event, the results obtained here with purified myosin and actin clearly revealed that the Mg$^{2+}$-ATPase activity of myosin from thyrotoxic rabbits was increased both in the presence and absence of actin. Moreover, the Mg$^{2+}$-ATPase was increased almost to the same extent as we observed previously for the Ca$^{2+}$-ATPase activity in rabbits given a similar dosage of thyroxine.

The mechanism for ATP hydrolysis in the presence of calcium and magnesium as activators is thought to be similar and, in myosin-N, both activities are stimulated by thiol modification. It seems somewhat surprising, therefore, that the Mg$^{2+}$-ATPase of myosin-T is enhanced by thiol modification. The Ca$^{2+}$-ATPase activity of myosin-T is not stimulated upon blocking the SH$_2$ thiols. This has led to suggestions that thyroxine may exert its effects either by blocking the SH$_2$ thiols or by inducing a conformational change similar to that produced by modification of these groups in normal myosin. The present results further emphasize the unique nature of the changes in activity induced by thyroxine and the lack of any exact analogy to thiol modification.

Since myosin is known to possess recognition site(s) necessary for the regulation of actomyosin, the actin-activated ATPase of myosin from normal and thyrotoxic rabbits was assayed in the presence of a complex of troponin and tropomyosin (Table 2). The calcium sensitivity of cardiac actomyosin when combined with the regulatory proteins has been well documented and the results obtained with myosin-N are consistent with these findings. TN-TM complex from skeletal muscle was used in these experiments because of the limited tissue available. Although troponin and tropomyosin isolated from skeletal muscle are somewhat different in electrophoretic mobility and amino acid composition from similar proteins found in heart, they seem to have the same effect on the Ca$^{2+}$-stimulated ATPase activity of desensitized cardiac actomyosin.

Myosin-T exhibited the expected increase in activity upon addition of CaCl$_2$, indicating that the site(s) necessary for regulation of actomyosin activity were fully functional. In both myosin-T and myosin-N, these sites appeared to include either the SH$_2$ thiols or the region near these groups, since thiol modification reduced the calcium sensitivity. Thus it would appear that the actin-activated ATPase activity of myosin-T is modulated by the TN-TM complex in the same manner as normal myosin.

According to current concepts of muscle contraction, the ATPase activity of myosin is related closely to the speed of contraction. Evidence for increased speed of myocardial contraction in thyrotoxic animals has been obtained in isolated atrium or intact heart of guinea pig, in cat papillary muscle preparations and in intact dog heart. Opposite effects have been found in hypothyroidism. The enhanced actomyosin ATPase activity of cardiac myosin from thyrotoxic rabbits reported here strongly supports the possibility that the change in enzymatic activity of myosin is causally related to the increased velocity of shortening. On the other hand, the physiological significance, if any, of the increased apparent affinity of myosin-T for actin is uncertain. At the time when thyroxine administration has produced activation of myosin ATPase activity and increased velocity of contraction, there is no evidence of greater force development per cross-sectional area, such as might be anticipated if the increased affinity were manifest as a larger number of strong interactions between myosin and actin.

Acknowledgments

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References

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