ADENOSINETRIPHOSPHATASE ACTIVITY OF MICROSOMES FROM RABBIT SKELETAL MUSCLE; ITS GENERAL ENZYMIC PROPERTIES AND RELATION TO PHYSIOLOGICAL FUNCTION OF MICROSOMES

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It is generally accepted that the granules isolated from homogenates of the skeletal muscle are essential for the relaxation of glycerol-extracted muscle fibers1-8). The electron micrograph taken by NAGAI et al.7) indicated that the granules with relaxing activity would be originated from the sarcoplasmic reticulum9).

On the basis of their morphological observations, PORTER et al.10) postulated that the impulse from the sarcolemma to contractile material conducts through the sarcoplasmic reticulum. Furthermore, possible roles of the endoplasmic reticulum in carbohydrate metabolism have been described by SIEKERVITZ11). These studies indicate the importance of the sarcoplasmic reticulum in muscle activity. Many experimental results have been presented on microsomes of liver cells, but little information has been reported on the enzymic and biochemical characteristics of microsomes of the skeletal muscle. Therefore, general enzymic properties of the ATPase of the microsomes isolated from rabbit skeletal muscle will be presented in the present paper, particularly in connection with the physiological function of the microsomes.

MATERIALS AND METHODS

Preparation of microsomes: The minced rabbit skeletal muscle (hind leg and back) was homogenized for 60 sec in a Waring Blender with 3 volumes of extraction solution containing 80 mM KCl, 20 mM histidine and 5 mM K$_2$-oxalate (pH 7.2). The

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Abbreviations: ATP, adenosinetriphosphate; ITP, inosinetriphosphate; ATPase, adenosinetriphosphatase; Mg-ATPase, Mg$^{++}$-activated adenosinetriphosphatase; Pi, inorganic ortho-phosphate; DNP, 2,4-dinitrophenol; Tris, tris-(hydroxymethyl)-aminomethane.
homogenates were centrifuged at 2,000 g for 15 min. The turbid supernatant was centrifuged at 12,400 g for 10 min. The resulting supernatant was finally centrifuged at 67,500 g for 40 min. to yield a microsomal fraction. The residue was once washed with 0.1 M KCl solution and then resuspended in 0.1 M KCl. In some cases (Fig. 5-7), the residue was resuspended without washing in a solution containing 0.1 M KCl and 1 mM K$_2$-oxalate. All the operations were performed at 0°-5°C.\cite{2,4}\.

**Measurement of ATPase activity:** ATPase activity was usually determined in the reaction mixture containing 66.7 mM Tris-acetate buffer (pH 7.0), 50 mM KCl, 1 mM MgCl$_2$, 1 mM ATP-Na$_2$ and a suitable amount of the microsomes suspension in a final volume of 3.0 ml. The tubes containing the reaction mixture were immersed in a 20° bath. After the equilibration of the temperature, the reaction was started by the addition of the substrate. After certain time intervals, the reaction was stopped by adding 2.0 ml of 5% trichloroacetic acid, and the orthophosphate liberated in an aliquots of the filtrate was determined by the Fiske-Subbarow method.\cite{13}

**Determination of protein:** The content of protein was determined by the micro-Kjeldahl method, using a factor of 6.25.

**Reagents:** ATP was a Sigma preparation. Other reagents used were commercial products of the best reagent grade available.

### RESULTS

#### I. Enzymic properties of ATPase of microsomes of the skeletal muscle

**a) Effects of divalent cations:** The activity of the ATPase of the microsomes varied from one preparation to another, and for most preparations it increased gradually on standing at 0° during several days.

As shown in Fig. 1, the ATPase of the microsomes was activated by the addition of Mg$^{++}$, Ca$^{++}$ or Mn$^{++}$. However, a low but definite activity was observed even when no divalent cation was added.

The optimum concentration of Mg$^{++}$ for the activity was nearly equal to the concentration of ATP; the ATPase activity decreased with the increase in concentration of Mg$^{++}$ higher than that of ATP. These properties were observed in the presence of 1.0, 2.0 and 3.0 mM ATP. At 1.0 mM ATP, the degree of activation by Mn$^{++}$ was decreased when the concentration of Mn$^{++}$ was higher than 0.3 mM (up to 10 mM).

The ATPase of the microsomes was activated by adding Ca$^{++}$ alone, and it increased with increase in Ca$^{++}$ concentration (Fig. 1-B), though, as well known, the Kielley-Meyerhof ATPase is not influenced by Ca$^{++}$\cite{14}. In the presence of 1.0 mM Mg$^{++}$, Ca$^{++}$ inhibited the ATPase, but the complete inhibition could not be obtained even when the concentration of Ca$^{++}$ was ten times as high as that of Mg$^{++}$.

**b) Influence of pH on Mg-ATPase of microsomes:** The optimum pH for the Mg-ATPase of the microsomes shifted depending on the ratio of Mg$^{++}$ concentration to ATP (Fig. 2). In the absence of Mg$^{++}$, the optimum pH was around 8.6. When both Mg$^{++}$ and ATP were present in equi-molar concentration, the optimum pH was about 8.2. In the presence of higher Mg$^{++}$ con-
Fig. 1. Effect of divalent cation on ATPase of microsomes. 0.29 mg protein/ml; reaction time, 5 min. (A); effect of Mg++ in presence of various ATP concentrations. •, 1 mM ATP; ○, 2 mM ATP; x, 3 mM ATP. (B); effect of Ca++ and Mn++ in presence of 1 mM ATP. •, Ca++; □, Mn++; x, Ca++ plus 1 mM Mg++.

Fig. 2. Influence of pH on Mg-ATPase of microsomes. Conditions were the same as stated in Fig. 1. Mg++ concentration in mM is given by x, nil; •, 1; ○, 2.5.
centration (the ratio of Mg$^{2+}$ to ATP was 2.5), the optimum pH was about 7.4, which is very close to that obtained on the Kielley-Meyerhof Mg-ATPase\textsuperscript{14}.  

c) Effects of various reagents on Mg-ATPase of microsomes: Effects of various reagents on the Mg-ATPase of the microsomes are summarized in Table I. Azide had little effect on the Mg-ATPase, whereas fluoride and

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration mM</th>
<th>Relative activity %</th>
</tr>
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<tbody>
<tr>
<td>NaN\textsubscript{3}</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>KF</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>96.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>86.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
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</tr>
<tr>
<td>Salyrgan</td>
<td>0.11</td>
<td>88.7</td>
</tr>
<tr>
<td></td>
<td>1.75</td>
<td>61.5</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>24.2</td>
</tr>
<tr>
<td>long chain fatty acids</td>
<td>0.01–0.3</td>
<td>activation</td>
</tr>
<tr>
<td>caffeine</td>
<td>5.0</td>
<td>no effect</td>
</tr>
<tr>
<td>DNP</td>
<td>0.1</td>
<td>100–130</td>
</tr>
</tbody>
</table>

Salyrgan had inhibitory effect. These results were in line with the observation on the Kielley-Meyerhof Mg-ATPase\textsuperscript{14}. Caffeine which inhibits the relaxing activity of the microsomes\textsuperscript{19}, had no effect on the Mg-ATPase, and DNP which also inhibits the relaxing activity of the microsomes\textsuperscript{13}, activated it only slightly. The degree of activation by DNP varied from one preparation to another (1.0–1.3 times). In the presence of Mg$^{2+}$, 0.1 mM DNP increased 2.0–2.6 times the ATPase of the mitochondria prepared from rabbit skeletal muscle by the method of Chappell and Perry\textsuperscript{12}.

The ATPase of the microsomes was activated by various long chain fatty acids (see next section). On the other hand, oleate inhibited the relaxing activity of the microsomes.

The acetone dried microsomes had little or no ATPase activity.

II. Some observations on ATPase in relation to physiological function of microsomes

a) Effects of various long chain fatty acids on ATPase of microsomes: It is known that long chain fatty acids influence the enzymic activity, respiration and structure of the mitochondria\textsuperscript{16,17}. Therefore, the effect of various long
chain fatty acids on the ATPase of the microsomes was examined. As shown in Fig. 3, in the presence and the absence of Mg$^{++}$, the ATPase of the microsomes was activated by long chain fatty acids, except for stearate, which inhibited the ATPase in the presence of Mg$^{++}$. Among the fatty acids (C$_{12}$-C$_{18}$) tested, myristate was the most strong activator. Oleate, which is an unsaturated C$_{18}$-fatty acids, activated the ATPase more strongly than stearate,

![Graph showing effect of long chain fatty acids on ATPase of microsomes.](image)

**Fig. 3.** Effect of long chain fatty acids on ATPase of microsomes. Microsomes (0.33 mg protein/ml.) were preincubated for 15 min. with long chain fatty acid at 20°C and then, Mg$^{++}$ and ATP were added successively to the mixture. Reaction times were 2.5 min. and 5 min. in the presence and the absence of Mg$^{++}$, respectively. Ordinate; relative value of ATPase activity. Abscissa; Concentration of agents in mM. Concentration of Mg$^{++}$, 1 mM for empty symbols and 0 mM for black symbols. ◀, myristate; △, laurtrate; ○, oleate; □, stearate. ◀, 0.1 mM myristate; △, 0.1 mM laurtrate; ○, 0.1 mM oleate; □, 0.1 mM stearate.

a saturated C$_{18}$-fatty acid. The optical density of the microsomes suspension (1.2 mg protein/ml) at 520 mÅ was gradually decreased to about 40 per cent of the initial value in the presence of 0.68 mM oleate. The ATPase activity of the microsomes seems to be closely connected with their structure.

b) Effects of monovalent cations and ouabain on Mg-ATPase of microsomes:

It has recently been reported that the activity of the Mg-ATPase of the granules and the microsomes isolated from nerve$^{16}$ and brain$^{19,20}$ is stimulated by Na$^+$ and that the Mg-ATPase of human erythrocytes is dependent on the
simultaneous presence of Na\(^+\) and K\(^{+21}\). As illustrated in Fig. 4, the ATPase activity of the microsomes of the skeletal muscle was slightly affected by monovalent cations, such as Na\(^+\) and K\(^+\). In the presence of 4.0 mM K\(^+\), the Mg-ATPase activity was decreased gradually with increasing Na\(^+\) concentrations from 2.0 mM to 70 mM. In the presence of 37.3 mM or 70.7 mM K\(^+\), Na\(^+\)

had no effect on the Mg-ATPase. In the presence of Na\(^+\) less than 30 mM, the Mg-ATPase activity was decreased by adding K\(^+\) whose concentration was higher than that of Na\(^+\) but in the presence of Na\(^+\) higher than 30 mM, it was unaffected by adding K\(^+\).

Ouabain (up to 30 \(\mu\)M) did not inhibit the Mg-ATPase of the microsomes (Fig. 4-B). Furthermore, in the presence of 5.3 mM K\(^+\) and 17.4 mM Na\(^+\), 10 \(\mu\)M of ouabain did not inhibit the ATPase of the microsomes in the absence of Mg\(^{++}\) or in the presence of 5.0 mM Ca\(^{++}\).

c) Effect of Ca\(^{++}\) on Mg-ATPase of microsomes in presence of oxalate: The relaxing activity of microsomes is inhibited by Ca\(^{++}\) but seems to be potentiated by oxalate\(^{1,4-8}\). Therefore, the effect of Ca\(^{++}\) on the Mg-ATPase of the microsomes were studied in more detail in the presence of oxalate.

**Fig. 5** shows the effect of Ca\(^{++}\) on the ATP splitting by the Mg-ATPase in the presence of oxalate. In the absence of Ca\(^{++}\), the rate was 0.072 \(\mu\)moles Pi/mg protein/min: when 0.45 \(\mu\)moles of Ca\(^{++}\)/mg protein was added 5.5 minutes after the start of the reaction, the rate rose to 0.23 \(\mu\)moles Pi/mg protein/min. The acceleration of the rate continued for about 3 min. (extra-splitting phase), then the rate dropped to the same level as that of control. The “extrasplit-
FIG. 5. Effects of Ca++, DNP and caffeine on Pi liberation from ATP by Mg-ATPase in presence of oxalate. Reaction mixture contained 86 mM K+, 4 mM Na+, 2 mM ATP, 2 mM Mg++, 2 mM oxalate, 66.7 mM Tris-acetate buffer (pH 7.0) and 0.66 mg of microsomes in a total volume of 2.0 ml. Temp. was at 20°. Arrows a and b indicate addition of 0.45 μmoles/mg protein of following reagents. ○, ●, Ca++; □, ■, DNP; △, ▲, caffeine. Empty symbols for time course after addition of reagent at the beginning of the reaction. Black symbols for time course after addition of reagents 5.5 min after start of reaction.

Fig. 5. Effects of Ca++ on Pi liberation from ATP by Mg-ATPase in presence of oxalate. Reaction mixture contained 36 mM K+, 4 mM Na+, 2 mM ATP, 2 mM Mg++, 2 mM oxalate, 66.7 mM Tris-acetate buffer (pH 7.0) and 0.66 mg of microsomes in a total volume of 2.0 ml. Temp. was at 20°. Arrows a and b indicate addition of 0.45 μmoles/mg protein of following reagents. ○, ●, Ca++; □, ■, DNP; △, ▲, caffeine. Empty symbols for time course after addition of reagent at the beginning of the reaction. Black symbols for time course after addition of reagents 5.5 min after start of reaction.

"Extrasplicing phase" was also observed when Ca++ was added before the addition of ATP. These results confirmed the phenomenon reported by Hassebach and Makino. However, they did not determine the dependence of the rate of Pi liberation and the length of the "extra-splitting phase" on the amounts of Ca++ added. Fig. 6 shows time course of Pi liberation in the presence of various concentrations of Ca++. The rate in the "extra-splitting phase" was decreased and the length of this phase was increased in the amounts of Ca++. The acceleration of Pi liberation persisted for 20 min, when Ca++ was 1.5 or 4.0 μmoles/mg protein. The relation between the amounts of extra splitting of ATP and Ca++ (Pi: Ca ratio) was calculated from the results shown in Fig. 6. The Pi: Ca ratio was 1.5 in the range of 0.09 to 0.61 μmoles Ca++/mg protein. In the presence of 0.36 to 0.61 μmoles Ca++/mg protein, the Pi: Ca ratio was in the range from 1.0 to 1.5 (mean value was 1.23) for four microsomes preparations.

In the absence of oxalate, the extra splitting of ATP was scarcely observed in the presence of 0.4 to 0.97 μmoles Ca++/mg protein.

The influence on the extra splitting of ATP of the reagents, which have
Effects on muscle activity, was investigated. Caffeine (1.0, 4.17 or 8.33 mM) and ouabain (0.01 or 2.0 mM) had no effect on the Pi liberation in the "extra-splitting phase". On the other hand, DNP showed a marked influence on the "extra-splitting phase". As shown in Fig. 7-A, in the presence of 0.45 µmoles Ca++/mg protein, the rate in the phase was decreased and the length of the phase was increased by 0.5, 5.0 or 10.0 mM DNP. In the presence of 5.0 mM DNP, the Pi: Ca ratio was 2.1; twice as high as that in the absence of DNP. Similar phenomena were observed in the presence of 0.36 or 0.58 µmoles Ca/mg protein and 5.0 mM DNP (Fig. 7-B).

The activity of Pi liberation in the "extra-splitting phase" was not influenced by changing the composition of the reaction mixture from the standard one to 72.3 mM Na+ and 12.8 mM K+.

Caffeine and DNP (0.15-5.0 mM) did not alter the time course of Pi liberation of ATP by the Mg-ATPase in the presence of oxalate, as shown in Fig. 5 for the case of the addition of 0.15 mM of the reagents.

When ITP was used as the substrate, the time course of Pi liberation from ITP was altered by adding Ca++ as was in the case of ATP, though the results were not so conclusive. There remains some possibility of the contamination of adenine nucleotide in ITP used and/or the presence of nucleoside diphosphokinase in the microsomes.
Fig. 7. Effect of DNP on extra splitting of ATP caused by addition of Ca++. Conditions were the same as stated in Fig. 5. (A): Ca++ was 0.45 μmoles/mg protein; DNP in mM was given by •, nil; ▲, 0.5; ●, 5.0; ■, 10. (B): DNP was 0 (○, □) or 5.0 mM (●, ■); amounts of Ca++ in μmoles/mg protein was given by ◊, 0.36; □, 0.58.

DISCUSSION

As described in "RESULTS I", the properties of the ATPase of the microsomes were similar to those of the Kielley-Meyerhof ATPase\(^\text{14}\), except that the ATPase of the microsomes was activated by Ca++ alone (Fig. 1-B). The activation by Ca++, was also reported on the ATPase of the microsomes of myometrium\(^\text{24}\) and the white muscle ATPase\(^\text{25}\) prepared according to the Kielley-Meyerhof procedure. It seems likely that the difference in the effect of Ca++ depends on the purity of the enzyme and on the kind of muscle from which the enzyme is isolated. The properties of the enzyme may also depend on the structure of the granules. Therefore, it is reasonably suggested that the ATPase of the microsomes of the skeletal muscle and the Kielley-Meyerhof one is the same enzyme and the small differences in their properties are due to the change in the structure of granules.
The Mg-ATPases isolated from the nerve and brain are stimulated by Na⁺, and are considered to be involved in the mechanism of active transport of Na⁺ across the cell membranes.¹⁸,²⁰ There are some similarities in the properties of the Mg-ATPase from crab nerve and that of the microsomes from the skeletal muscle. Both enzymes are isolated as associated with submicroscopic granules, show optimum activity at pH 7.2 (nerve) and 7.4 (muscle), and are inhibited by Ca++. There is, however, a difference in the effect of Na⁺ on these enzymes; Na⁺ activates the Mg-ATPase of crab nerve but it inhibits the enzyme of the microsomes of the muscle only slightly. This may be attributed to either that the microsomes of the muscle already contain Na⁺ enough to activate the Mg-ATPase or that the Mg-ATPase is not substantially activated by Na⁺.

From the effects of some reagents⁷,¹² on the relaxing activity and the Mg-ATPase (TABLE I), it may be concluded that there is no direct correlation between the ATPase and the relaxing activities of the microsomes. The extra splitting of ATP reported by Hasselbach and Makinose,²² was clearly observed in the presence of oxalate. In the absence of oxalate, however, the extra splitting of ATP was scarcely observed. When Ca++, caffeine or DNP is added to microsomes, the relaxing activity is inhibited and then spontaneously recovers²²,²³. Hasselbach and Makinose have suggested that the "Ca-pump" plays a role in the recovery from the Ca-inhibition. However, the mechanism of the recovery from the caffeine- or the DNP-inhibition may be different from the one from the Ca-inhibition because, as described above, caffeine and DNP did not accelerate the splitting of ATP by the microsomes. It seems likely that caffeine could not inhibit the "Ca-pump" of the microsomes, even though caffeine inhibited the relaxing activity. Therefore, it seems reasonable to conclude that the relaxing activity of microsomes can not be explained only by the absorption of Ca++.  

SUMMARY

The microsomal fraction of rabbit skeletal muscle was isolated and its ATPase activity was studied, in connection with the physiological function of the microsomes. The following results were obtained.
1. From its enzymic properties, it was suggested that the microsomal ATPase is the same as the Kielley-Meyerhof one.
2. The ATPase was activated by several long chain fatty acids, slightly affected by Na⁺ or K⁺ and not inhibited by ouabain.
3. In the presence of oxalate, the extra splitting of ATP by the addition of Ca++ was confirmed. The rate of ATP hydrolysis in the "extra-splitting phase" was decreased and the length of this phase was increased with increase in the amount of Ca++. The extra splitting of ATP was not affected by the
preincubation of the microsomes with ATP. The extra splitting of ATP was scarcely affected by caffeine and ouabain, while DNP modified remarkably this ATP splitting.

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REFERENCES