Inhibition of Actomyosin Subfragment 1 ATPase Activity by Analog Peptides of the Actin-binding Site around the Cys(SH1) of Myosin Heavy Chain*

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The synthetic heptapeptide, Ile-Arg-Ile-Cys-Arg-Leu-Gly-ethoxy, an analog of one of the actin binding sites on myosin head (S-site) (Suzuki, R., Nishi, N., Tokura, S., and Morita, F. (1987) J. Biol. Chem. 262, 11410–11412) was found to completely inhibit the acto-S-1 (myosin subfragment 1) ATPase activity. The effect of the heptapeptide on the binding ability of S-1 for F-actin was determined by a ultracentrifugal separation. Results indicated that the heptapeptide scarcely dissociated the acto-S-1 complex during the ATPase reaction. Consistent results were obtained from the acto-S-1 ATPase activities determined as a function of S-1 concentrations in the absence or presence of the heptapeptide at a fixed F-actin concentration. The heptapeptide reduced the maximum acto-S-1 ATPase activity without affecting the apparent dissociation constant of the acto-S-1 complex. The heptapeptide bound by a site on actin complementary to the S-site probably inhibits the activation of S-1 ATPase by F-actin. These results suggest that S-1 ATPase is necessary to rebind transiently with F-actin at the S-site in order to be activated by F-actin. This is consistent with the activation mechanism proposed assuming the two actin-binding sites on S-1 ATPase (Katoh, T., and Morita F. (1984) J. Biochem. (Tokyo) 96, 1223–1230).

Muscle contraction is driven by a cyclic attachment and detachment of myosin and actin coupled with actomyosin ATPase cycle. Although the kinetic mechanisms of actomyosin ATPase are well understood (1–3), the structural information coupled with each reaction step of the ATPase remains elusive. In order to investigate the correlation between the structural state and the kinetic state of actomyosin ATPase, the interaction sites of myosin and actin must be defined.

Several lines of evidence gathered mainly by limited proteolysis of S-1 by proteolytic enzymes and chemical crosslinking between S-1 and F-actin with bifunctional reagents suggest that the actin-binding site of myosin is located on the peptide region connecting the 50- and 20-kDa domains of S-1 heavy chain (4–6). Katoh et al. (7–9) showed that there was another actin-binding site located in a region around the reactive Cys(SH1) and Cys(SH2) on S-1 heavy chain. They proposed that S-1 had at least two actin-binding sites, one around the reactive Cys(SH1) and Cys(SH2) (S site) and the other around the junction between the 50- and 20-kDa domains (J site). The S and J sites were assumed to determine the high affinity of acto-S-1 rigor complex and the low affinity in the presence of ATP, respectively. The activation of S-1 ATPase by F-actin was assumed to be induced by a transient attachment of the S site with F-actin after formation of the reaction products at the ATPase site (8).

The amino acid sequence around the Cys(SH1) and Cys(SH2) is highly conserved among myosins from various sources (10–19). The amino acid sequence of Ile-Arg-Ile adjacent Cys(SH1) toward the N-terminal was assumed to be a major locus in the S site based on the comparison of affinities of various synthetic peptides with F-actin (20, 21). Among several synthesized peptides, the heptapeptide Ile-Arg-Ile-Cys(SH1)-Arg-Lys-Gly-OEt was the smallest one having the highest affinity for F-actin (20, 21).

In this paper, we report that acto-S-1 ATPase activity is completely inhibited by the synthetic heptapeptide and more weakly by tripeptide, Ile-Arg-Ile-OEt. We also note that the type of inhibition by the heptapeptide is consistent with the model on actomyosin ATPase proposed previously (8).

EXPERIMENTAL PROCEDURES

Materials—Sodium salt of ATP, bovine serum albumin, and α-chymotryptsin were purchased from Sigma. t-Butoxycarbonyl (Boc) amino acids were obtained from either Peptide Institute Inc. or Kolusan Chemical Works Ltd. Diphenyl phosphoramide was obtained from Daiichi Pure Chemicals Co. Ltd.

Proteins—Rabbit skeletal muscle myosin was prepared by the method of Perry (22) and the fraction precipitated between 37.5–42.5% saturation of ammonium sulfate was used.

Rabbit skeletal myosin S-1 was prepared by α-chymotryptic digestion of myosin according to Onodera and Yagi (23). Only S-1(a1) isoenzyme isolated after DEAE-Sepharose CL6B column chromatography was used throughout this work. Before use, S-1 was clarified by centrifugation at 214,000 × g for 15 min at 4 °C. Rabbit skeletal actin was prepared by a modification of the method of Spudich and Watt (24). Rabbit skeletal heavy meromyosin prepared by α-chymotryptic digestion of myosin was donated by Dr. Michiharu Yoshida.

Peptide Synthesis—Peptides were synthesized by a liquid-phase procedure described previously (20, 21). α-Amino groups of amino acid corresponding to the N-terminal end were protected with Boc group. Carboxyl groups of amino acid corresponding to the C-terminal end were protected as either ethoxy (OEt) or methoxy (OMe) group. Side chains were protected with mesitylenesulfonyl group at the guanidyl group of Arg, methoxybenzyl group at the sulhydryl group of Cys, and benzoxycarbonyl group at the ε-amino group of Lys. Protected Boc-Ile-Arg-Ile-OEt and Boc-Cys-Arg-Lys-Gly-OEt were synthesized stepwise using diphenylphosphoryl azide as the coupling

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‡ The abbreviations used are: S-1, myosin subfragment-1; OMe, methoxy; OEt, ethoxy; Boc, t-butoxycarbonyl.
weights used for myosin, S-l, heavy meromyosin, and actin monomer by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Yajima et al. (26).

The deprotected heptapeptide (40-50 mg) was dissolved in 2 ml of 0.5 M Tris-HCl, pH 8.0, and dithiothreitol was added to 0.1 M. After standing for 10-12 h at room temperature, iodoacetamide was added to 0.2 M to protect the sulfhydryl group. After standing for 1 h further at room temperature, β-mercaptoethanol was added to 0.4 M. The peptide was applied on a Sephadex G-15 column equilibrated with 1 M acetic acid. The elution pattern monitored by absorbance at 240 nm showed three peaks. Quantitative amino acid analysis indicated that both first and second peak components were heptapeptide, and the third peak contained uncoupled peptide fragments. The first peak fractions were used after lyophilization. The deprotected tripeptide, Ile-Arg-Ile-OMe, was converted to CTF form by passing through an anion exchanger, Amberlite IRA 400 column and then lyophilized. When used, the lyophilized peptides were dissolved in H_2O and the concentrations were determined by quantitative amino acid analysis. The amino acid analysis was performed by a JASCO Amino Acid Analysis System OPA, after hydrolysis for 24 h at 110 °C in 6 M HCl.

**ATPase Activities—ATPase activities of S-1 in the presence or absence of 1.6-2.0 μM F-actin were measured at 0.70-4.8 μM S-1 and in the presence of peptide, from 0 to 5 mM. Acto-S-1 ATPase activity as a function of S-1 concentrations (0.3-30 μM) was determined in the presence of 1.0 μM F-actin, and the ATPase activity of S-1 alone measured at identical S-1 concentration was subtracted from the total activity. Conditions were 20 mM Tris-HCl, pH 8.0, 2 mM ATP, 2 mM MgCl_2, and 0.1 mM bovine serum albumin at 25 °C. The ionic strength was adjusted to 10 mM by addition of KCl. The ATPase activity was determined from the time course of P liberated, and the concentration of F, was determined by the method of Ohnishi et al. (27, 28).

Concentrations of myosin, S-1, heavy meromyosin, and actin were determined spectrophotometrically using the absorption coefficients of ε^290 (at 290 nm) = 5.4, 7.9, 6.35, and 11.0, respectively. Molar weights used for myosin, S-1, heavy meromyosin, and actin monomer were 480,000, 115,000, 365,000, and 42,000, respectively.

**RESULTS**

Inhibition of Acto-S-1 ATPase Activity by Ile-Arg-Ile-Cys-Arg-Lys-Gly-OEt—As shown in Fig. 1, the heptapeptide inhibited the acto-S-1 ATPase activity at 10 mM ionic strength. Fifty % inhibition was observed at 0.07 mM heptapeptide. The value of acto-S-1 ATPase activity in the presence of 0.5 mM heptapeptide was almost equal to that of S-1 ATPase activity in the absence of F-actin. As shown by the solid circles in Fig. 1, the heptapeptide did not inhibit the ATPase activity of S-1 alone. These results indicate that the heptapeptide inhibits the activation of S-1 ATPase by F-actin, but does not directly affect the ATPase site of S-1.

**Effect of Heptapeptide on the Binding of S-1 with F-actin in the Presence of ATP**—In order to know whether the inhibition of acto-S-1 ATPase by the heptapeptide is due to a dissociation of the acto-S-1 by the heptapeptide or not, effects of the

\[
\begin{align*}
\text{Equation 1} & : & V_n &= V_{\text{SM}} + \frac{V_n}{k_d} + \frac{V_{\text{SM}}}{k_d} \\
\text{Equation 2} & : & V_n &= V_{\text{SM}} + \frac{V_n}{K_d} \\
\text{Equation 3} & : & \frac{V_n}{K_d} &= \frac{V_{\text{SM}}}{K_d} \\
\end{align*}
\]

where A is an actin monomer, M is S-1 saturated with ATP, P is the heptapeptide, and AM is the steady state intermediate species of acto-S-1 ATPase. K_d and k_d are dissociation constants of each molecular species and the turnover rate constant of the acto-S-1 ATPase, respectively. If only AM is activated by F-actin, but not the heptapeptide-AM ternary complex, PAM, the ATPase activity at the steady state is given by

\[
V_n = \frac{V_{\text{M}}}{K_n + [M]} \\
\]

and

\[
V_n = \frac{V_{\text{M}}}{1 + \frac{[P]}{K_d}} \\
\]

where \(V_{\text{M}}\) is the total concentration of the actin monomer used. Equation 2 is similar to Michaelis-Menten equation, and the plot of \(V_n\) versus [M] gives a hyperbola whose maximum activity, \(V_{\text{M}}\), is obtained from \(V_n\) at infinite [M], and \(K_n\) is [M] to give \(V_n/2\). Only \(V_n\) is dependent on [P], and \(K_d\) is calculated from Equation 3 using [P] and \(V_{\text{M}}\) which is \(V_n\) at [P] = 0.

**FIG. 1. Inhibition of acto-S-1 ATPase Activity by Ile-Arg-Ile-Cys-Arg-Lys-Gly-OEt**—The open circles indicate the acto-S-1 ATPase activity. The concentrations of S-1 and F-actin were 0.7 and 1.6 μM, respectively. The solid circles indicate the ATPase activity of S-1 alone. The S-1 concentration was 4.8 μM. Other conditions: 2 mM ATP, 2 mM MgCl_2, 0.1 mM bovine serum albumin, and 20 mM Tris-HCl, pH 8.0, at 25 °C. The ionic strength was adjusted to 0 mM by adding KCl.
heptapeptide on the binding of S-1 with F-actin were examined. Acto-S-1 were ultracentrifuged at 214,000 \times g for 15 min at 25 °C after the addition of 2 mM MgATP. Based on the determination of the P_i concentration after centrifugation, 0.8 mM ATP still remained even in the absence of the peptide under which ATPase activity is the highest. The pellet components were applied on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the amount of S-1 heavy chain in the pellet was determined by densitometry. Table I lists concentrations of S-1 bound with F-actin measured at three different concentrations of S-1 and F-actin in the absence and presence of various concentrations of heptapeptide. Results show that the concentration of S-1 bound with F-actin essentially was not affected by the presence of the heptapeptide. Table I(a) shows results obtained under identical conditions to those of Fig. 1. The acto-S-1 was not significantly dissociated by the heptapeptide even at such a low concentration of F-actin. These results suggest that the inhibitory action of the heptapeptide for the activation of S-1 ATPase by F-actin is not due to a dissociation of acto-S-1 complex.

In order to determine in more detail the manner of inhibitory action of the heptapeptide, acto-S-1 ATPase activity was measured as a function of S-1 concentrations at 1.0 μM F-actin in the absence and presence of various concentrations of heptapeptide. The ATPase activity is expressed as ATP turn over rate/actin monomer. As shown in Fig. 2, the ATPase activity showed a good fit to simple saturating kinetics with respect to S-1 concentration in the presence of a heptapeptide lower than 0.1 mM. Data were analyzed according to Equations 2 and 3. The values of \( V_{\text{m}} \), \( K_{\text{m}} \), and \( K_d \) determined are summarized in Table II. Both \( V_{\text{m}} \) and \( K_{\text{m}} \) values in the absence of heptapeptide agree with the respective value obtained from the S-1 ATPase activity determined as a function of S-1 concentration in the presence of a heptapeptide.

In the presence of heptapeptide higher than 0.2 mM, data deviated from a simple hyperbola and showed a tendency toward positive cooperativity (curve d of Fig. 2). With increases in the heptapeptide concentration, \( K_d \) may become smaller than \( K_i \). There may also be an interaction among different S-1 molecules bound on a F-actin filament during the steady state of ATPase, and the heptapeptide bound on the F-actin may enhance the interaction.

**TABLE I**

Effect of the heptapeptide, Ile-Arg-Ile-Cys-Arg-Lys-Gly-OEt, on the binding between S-1 and F-actin in the presence of ATP

<table>
<thead>
<tr>
<th>Conc. of heptapeptide (mM)</th>
<th>Bound S-1 with F-actin (PM)</th>
<th>0.7 μM S-1</th>
<th>0.8 μM S-1</th>
<th>0.4 μM S-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.072 ± 0.002 (9)</td>
<td>0.50 ± 0.04 (15)</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.082 ± 0.003 (3)</td>
<td>0.50 ± 0.11 (5)</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.094 ± 0.010 (3)</td>
<td>0.60 ± 0.11 (5)</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.098 ± 0.014 (3)</td>
<td>0.49 ± 0.09 (5)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.040 ± 0.006 (3)</td>
<td>0.46 ± 0.14 (5)</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>0.040 ± 0.006 (3)</td>
<td>0.46 ± 0.14 (5)</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

* Results from a single experiment.

**TABLE II**

The dissociation constant of heptapeptide-F-actin complex, \( K_{o} \), was calculated from Equation 3.

<table>
<thead>
<tr>
<th>Conc. of heptapeptide (μM)</th>
<th>( K_{o} ) (μM)</th>
<th>( V_{m} ) (PM)</th>
<th>( K_{d} ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>15</td>
<td>16</td>
<td>46</td>
</tr>
<tr>
<td>0.05</td>
<td>16</td>
<td>7.7</td>
<td>46</td>
</tr>
<tr>
<td>0.10</td>
<td>14</td>
<td>5.2</td>
<td>48</td>
</tr>
</tbody>
</table>

**Inhibitory Effect of Ile-Arg-Ile-OMe on Activation of S-1 ATPase by F-actin**—The activation of S-1 ATPase by F-actin was also inhibited by the tripeptide, Ile-Arg-Ile-OMe, as shown in Fig. 3. Fifty % inhibition was observed at 1.8 mM peptide. The tripeptide also did not inhibit the ATPase activity of S-1 alone (Fig. 3). Furthermore, the acto-S-1 complex in the presence of ATP could not be dissociated by the tripeptide (Table III). The manner of the inhibitory action of the tripeptide is essentially identical to that of the heptapeptide, although much higher peptide concentrations were
needed than those of the heptapeptide.

Inhibitory Effect of Heptapeptide on Myosin and Heavy Meromyosin ATPases in the Presence of F-actin—Both actomyosin and acto-heavy meromyosin ATPases were also inhibited by the heptapeptide (Fig. 4). The concentration of heptapeptide at 50% inhibition was 0.28 mM with myosin and 0.05 mM with heavy meromyosin. Myosin is a filamentous form under these conditions and the affinity of myosin for F-actin, in vivo, however, is probably more intricate, because myosin slides F-actin as activating the ATPase activity. There should be several other contact regions which have stationary or transient interactions with F-actin. Recently, Muhlrad (30) reported that the renatured N-terminal 27-kDa fragment of S-l heavy chain also binds F-actin with ATP-sensitive manner.

Mitchell et al. (31) reported that the 10- to 12-kDa peptide containing only the Cys(SH1), but not Cys(SH2), was prepared from S-1 by Staphylococcus aureus V-8 protease cleavage, and this 10-12 kDa peptide retained the ability to bind both the F-actin and the light chain. Katoh et al. (32) reported that the A1 light chain increased the affinity of isolated 20-

**TABLE III**

<table>
<thead>
<tr>
<th>Conc of tripeptide (mM)</th>
<th>Bound S-l with F-actin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.12 ± 0.01 (14)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.10 ± 0.01 (5)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.11 ± 0.01 (6)</td>
</tr>
<tr>
<td>2.5</td>
<td>0.12 ± 0.01 (4)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.16 ± 0.02 (6)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

As reported in the previous papers, the heptapeptide used in the present study is the smallest S-site analog having the highest affinity for F-actin (20, 21). The heptapeptide inhibited the acto-S-1 formation competitively to S-1 in the absence of ATP (20, 21). In the present study, we examined the effects of the heptapeptide on acto-S-1 ATPase activity and on the affinity between S-1 and F-actin during the ATPase. In contrast to the acto-S-1 rigor complex, there was almost no effect by the presence of the heptapeptide, on the affinity of acto-S-1 complex during the ATPase, although the ATPase activity was strongly inhibited (Fig. 1 and Table I). As shown in Fig. 1, the heptapeptide, however, did not inhibit the ATPase activity of S-1 alone. These results indicate that the heptapeptide inhibits the activation of S-1 ATPase by F-actin, but does not directly affect the S-1 ATPase site.

As shown in Fig. 2 and Table II, the acto-S-1 ATPase activities determined as a function of S-1 concentrations were consistent with the scheme of Equation 1 where the ATPase of heptapeptide-AM ternary complex is not activated by F-actin. These results are also consistent with those of Fig. 1 and Table I. Based on kinetic studies, the activation of ATPase by F-actin is due to an acceleration in the release of ATPase products (1-3). Since the heptapeptide probably binds to a complementary site on actin monomers against the S-site, the results presented in this paper suggest that the S-site on S-1 is necessary to bind with the site on actin transiently to accelerate the release of products. Furthermore, the site on S-1 keeping the affinity with F-actin during the steady state ATPase is thought to be different from the site necessary to activate the release of products. These results are consistent with the Katoh and Morita (8) model in which the J site keeps acto-S-1 link at the steady state of ATPase, and the rebinding of S site with F-actin is necessary to activate the release of products. The interaction between myosin and F-actin, in vivo, however, is probably more intricate, because myosin slides F-actin as activating the ATPase activity. There should be several other contact regions which have stationary or transient interactions with F-actin. Recently, Muhlrad (30) reported that the renatured N-terminal 27-kDa fragment of S-1 heavy chain also binds F-actin with ATP-sensitive manner.

Mitchell et al. (31) reported that the 10- to 12-kDa peptide containing only the Cys(SH1), but not Cys(SH2), was prepared from S-1 by Staphylococcus aureus V-8 protease cleavage, and this 10-12 kDa peptide retained the ability to bind both the F-actin and the light chain. Katoh et al. (32) reported that the A1 light chain increased the affinity of isolated 20-
kDa fragment of S-1 for F-actin. There are several reports that actin interacts with A1 at the N-terminal residues (33-35). In the case of S-1(A1) isoenzyme at least, the tertiary structure around the S-site may, therefore, be constructed with both the peptide region around the Cys(SH1) of S-1 heavy chain and the N-terminal region of A1 light chain.

Apparent dissociation constant of the heptapeptide with F-actin, $K_d$, was calculated as about 47 $\mu$M from the acto-S-1 ATPase activity (Table II). This value is about one-fourth that obtained previously from the degree of inhibition of the heptapeptide against the formation of acto-S-1 rigor complex (20, 21). The discrepancy may partly be due to the difference in temperature which is 25°C in the present experiment in contrast to 4°C in the previous study.

The primary structure around the Cys(SH1) and Cys(SH2) is strongly conserved among various myosins (10-10). The homology of structure seems to indicate that the region plays an important role in physiological function. It is interesting to note a possible role of the S site on the Ca$^{2+}$ regulatory function. Chalovich and Eisenberg (36) reported that the inhibition of the acto-activated ATPase caused by troponin-tropomyosin in the absence of Ca$^{2+}$ was mainly due to a decrease in the rate of a kinetic step but not a decrease in affinity between myosin and actin. Furthermore, they showed that troponin-tropomyosin inhibited the binding between myosin and F-actin in the strong binding state that is rigor complex or complex in the presence of ADP, but not the weak binding state. The manner of troponin-tropomyosin in the absence of Ca$^{2+}$ is thus similar to that of the heptapeptide which inhibits the formation of rigor complex but does not affect the complex formation in the presence of ATP. There is a possibility, therefore, that troponin-tropomyosin in the absence of Ca$^{2+}$ inhibits the binding of F-actin at the S site such as to cover the site on actin, resulting in the inhibition of the release of products. If this is so, the low affinity between actin and myosin kept at the $J$ site in the presence of ATP should hardly be affected by Ca$^{2+}$. This hypothesis seems to provide a natural explanation for the Ca$^{2+}$ regulation of troponin-tropomyosin.

Previous studies using synthetic peptides indicated that the sequence of Ile-Arg-Ile adjacent to the N-terminal side of Cys(SH1) is an important locus for binding with F-actin (20, 21). As shown in Fig. 3 and Table III, the tripeptide, Ile-Arg-Ile-OMe, also inhibited the activation of S-1 ATPase by F-actin and F-actin and F-actin in the strong binding state that is rigor complex or complex in the presence of ADP, but not the weak binding state. The manner of troponin-tropomyosin in the absence of Ca$^{2+}$ is thus similar to that of the heptapeptide which inhibits the formation of rigor complex but does not affect the complex formation in the presence of ATP. There is a possibility, therefore, that troponin-tropomyosin in the absence of Ca$^{2+}$ inhibits the binding of F-actin at the S site such as to cover the site on actin, resulting in the inhibition of the release of products. If this is so, the low affinity between actin and myosin kept at the $J$ site in the presence of ATP should hardly be affected by Ca$^{2+}$. This hypothesis seems to provide a natural explanation for the Ca$^{2+}$ regulation of troponin-tropomyosin.

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