Effect of Ethylene Glycol and Ca\(^{2+}\) on the Binding of Mg\(^{2+}\)·Adenyl-5'-yl Imidodiphosphate to Rabbit Skeletal Myofibrils

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The binding of Mg\(^{2+}\)-adenyl-5'-yl imidodiphosphate (Mg\(^{2+}\)-AMP-PNP) to rabbit skeletal myofibrils has been measured in aqueous solution and in 50% ethylene glycol in the presence and absence of Ca\(^{2+}\). In water, the observed binding was weak with less than half the calculated myosin active sites filled even at 1 mM Mg\(^{2+}\)-AMP-PNP. In 50% ethylene glycol, the binding is at least 100-fold tighter and extrapolates to the expected number of binding sites. This is contrasted to the small change seen for Mg\(^{2+}\)-ADP binding between the same sets of conditions. This difference between Mg\(^{2+}\)-AMP-PNP and Mg\(^{2+}\)-ADP is attributed to the strong coupling of Mg\(^{2+}\)-AMP-PNP binding to dissociation of myosin cross-bridges. The Ca\(^{2+}\) sensitivity of Mg\(^{2+}\)-AMP-PNP binding in 50% ethylene glycol is taken as further evidence of the thermodynamic coupling of Mg\(^{2+}\)-AMP-PNP binding to cross-bridge dissociation. In addition, the binding of Mg\(^{2+}\)-AMP-PNP in 50% ethylene glycol is biphasic while Mg\(^{2+}\)-ADP binding under the same conditions is not. The biphasic Mg\(^{2+}\)-AMP-PNP binding could be caused by either the presence of two or more classes of cross-bridges or by negative cooperativity, but the presence of only a single class of Mg\(^{2+}\)-ADP-binding sites implies that if multiple classes of sites are involved, they do not simply differ in steric hindrance or accessibility of the binding site as a whole. The importance of using purified AMP-PNP in the study of actomyosin-AMP-PNP complexes is discussed.

Skeletal muscle fibers contain arrays of interdigitating filaments of two types: polymerized myosin and polymerized actin. These filaments can touch each other via radial extensions of the myosin filament called cross-bridges, which each contain an active site where ATP is hydrolyzed to supply the energy necessary for contraction. The driving force of muscle contraction is thought to result from a change in the structure and/or orientation of the cross-bridge while attached to the actin filament. The only well-characterized attached crossbridge state is the rigor state seen in the absence of nucleotide. X-ray diffraction and electron microscopic studies indicate that in rigor the cross-bridges uniformly assume an angle of about 45° relative to the actin filament. Since other attached crossbridge states exist only transiently in the presence of ATP, attention has focused on product and substrate analog complexes with cross-bridges which may have a second conformation different than that in rigor.

Addition of one of the products of ATP hydrolysis, Mg\(^{2+}\)-ADP, to muscle fibers does not change their stiffness (4, 5), indicating that its complex with the myosin cross-bridge remains attached to actin. There is some evidence that the Mg\(^{2+}\)-ADP complex is different from rigor (5-8), but since the x-ray diffraction pattern is identical to rigor (9) the changes have been assumed (8) to be local rather than changing the orientation of the cross-bridge relative to the actin filament.

More attention has been focused on the Mg\(^{2+}\)-AMP-PNP cross-bridge complex. This nucleotide analog of ATP also does not change fiber stiffness (10, 11), but several spectroscopic and other techniques (5, 7, 8, 10, 12-16) have provided convincing evidence that it does cause a change in the orientation of at least half of the cross-bridges. There is considerable disagreement, however, on whether these changed cross-bridges are still attached to actin (7, 12, 13, 17).

Tregear et al. (11) have recently found, however, that in 50% ethylene glycol, rabbit psoas muscle became completely relaxed with the stiffness dropping to very low values when bathed in 1 mM Mg\(^{2+}\)-AMP-PNP. It was later shown by Marston and Tregear (18) that the primary effect of substituting 50% ethylene glycol for water was to weaken the affinity of myosin for actin by a factor of 100, apparently just enough to allow 1 mM Mg\(^{2+}\)-AMP-PNP to dissociate all the cross-bridges. They found that there was no change in the affinity of nucleotides for myosin alone.

In this paper, I show that there is an increase by a factor of greater than 100 in the affinity of Mg\(^{2+}\)-AMP-PNP for rabbit skeletal myofibrils in going from water to 50% ethylene glycol and that there is clear evidence for biphasic binding and sensitivity to Ca\(^{2+}\). None of these effects, however, are seen with Mg\(^{2+}\)-ADP.

EXPERIMENTAL PROCEDURES

Myofibrils were prepared from rabbit back muscles or bovine ventricles by the method of Siemankowski and Dreizen (19) and stored before use in 50% glycerol at -20 °C and appeared to be fully overlapped in the phase-contrast microscope. ADP (dicyclohexyl ammonium salt) and Ap,A were obtained from Sigma and used without purification.

AMP-PNP was obtained from Sigma and Boehringer Mannheim and, when analyzed by isotachophoresis on an LKB instrument, was found to contain at least 1% of a slower moving impurity (presumably AMP-PN (29)) besides smaller amounts of other impurities. Initial attempts to purify AMP-PNP involved chromatography on

The abbreviations used are: AMP-PNP, adenylyl-5'-yl imidodiphosphate; AMP-PN, adenylyl-5'-yl phosphoramidate; Ap,A, P1', P1, diadenosine 5'-pentaphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid.
Whatman DE52 using an 0.01-0.4 M NH4HCO3 salt gradient (pH 8) followed by lyophilization of the peak. When the lyophilized material was dissolved in a small volume of water, its pH was 4 and there was still about 3% of the major impurity left when checked by isotachophoresis. The purity of the material before lyophilization could not be determined because the presence of NH4HCO3 causes bubbles to form in the capillary of the isotachophoresis instrument. When the same conditions for DE52 chromatography were used except for a NaCl salt gradient (with 0.5 m Tris (pH 8)), the material in the major peak was greater than 99% pure by isotachophoresis both before and after lyophilization.

The experiments in this paper were done with AMP-PNP purified in 30-mg batches on Whatman DE52 (1.5 x 50 cm) using a linear gradient of 500 ml of 0.1 M Tris-DEDC (pH 8) versus 500 ml of 0.1 M Tris-HCl, 0.3 M NaCl. The major peak was dialyzed for 90 min (>95% removal of monovalent salt with about 10% loss of AMP-PNP) and then lyophilized and used within 24 h.

Nucleotide binding was measured by a centrifuge method as previously described (21) in either aqueous or 50% (v/v) ethylene glycol solutions of 50 mM KCl, 30 mM Tris, 5 mM HEPES (pH 7.5), 10^{-4} M CaCl2, 5 mM MgCl2, 2 mM NaN3, using myofibril suspensions containing about 50 mg/ml total protein. For experiments without Ca++, 10^{-4} M EGTA was substituted. Full saturation of the myofibril-binding sites resulted in a decrease in the A_{550} of the supernatant of about 0.5 absorbance unit. The pH of glycol-containing buffers was adjusted to 7.5 before being diluted 2:1 with ethylene glycol. The pH meter reading for the diluted buffer was 7.4. In control experiments, no significant time dependence to the amount of Mg$^{2+}$-AMP-PNP binding was found.

The myosin content of myofibrils was determined by dissolving them in 5% sodium dodecyl sulfate and using the extinction coefficient previously determined (21). Nucleotide concentrations were determined by using ε_{260} = 15,400.

RESULTS

In a previous publication (21), I used a centrifuge method equivalent to equilibrium dialysis to measure Mg$^{2+}$-ADP binding to cardiac and skeletal myofibrils. I found that greater than 90% of the binding could be fit with a single binding constant very close to that seen for actin-myosin subfragment 1 complex. In this report, I have used the same technique to measure Mg$^{2+}$-AMP-PNP binding.

Fig. 1 shows the results of adding up to 1 mM Mg$^{2+}$-AMP-PNP to rabbit skeletal myofibrils at 0°C in aqueous buffer. The solid line represents the binding of Mg$^{2+}$-ADP reported previously by us under the same conditions (21). It is difficult to compare these data exactly because the ADP binding curve was corrected for nonspecific binding at high concentrations which may be different for AMP-PNP. However, it is clear that the degree of binding of AMP-PNP is only a fraction of that seen with ADP under the same conditions and appears to be no more than half-saturated at 1 mM concentration. There also appears to be about 5% of the sites which bind AMP-PNP tightly and are interpreted to represent the binding by myosin heads not attached to actin filaments.

The data in Fig. 1 were obtained with purified AMP-PNP. Commercially available AMP-PNP and/or AMP-PNP which has been lyophilized in the presence of volatile buffer salts contains a significant amount of an impurity which is apparently AMP-PN (20) (see "Experimental Procedures"). When unpurified AMP-PNP is used to titrate myofibrils in aqueous buffer, the impurity appears to be preferentially bound. This is illustrated by the data in Table I where, in titrations of cardiac myofibrils using 90 and 96% pure AMP-PNP, the apparent amount of bound AMP-PNP correlates with the amount of added impurity rather than the concentration of free AMP-PNP. Although these data do not prove that both the impurity and AMP-PNP bind to the same site, it does point out the importance of using purified AMP-PNP in these experiments.

Fig. 2 shows the results of adding Mg$^{2+}$-ADP to rabbit skeletal myofibrils at 0°C in 50% ethylene glycol. As with the Mg$^{2+}$-ADP binding results reported previously in aqueous solution (21), about 90% of the binding sites can be characterized by a single binding constant. Approximately 9% of the calculated amount of myosin heads bind Mg$^{2+}$-ADP very tightly and are interpreted to represent the binding by heads not attached to actin filaments. The dashed line represents a linear term used to fit the data at high concentrations of ADP and presumably represents a relatively minor amount of weak, nonspecific binding. The only apparent change between water and glycol is an increase in the dissociation constant by a factor of 3 (Table II).

The results with Mg$^{2+}$-AMP-PNP, however, are quite different in glycol than in water. Fig. 3 shows the binding curve in 50% ethylene glycol in the presence and absence of Ca++. Not only is the binding considerably tighter than in water, but there is now a relatively well-defined end point at about 80% of the calculated amount of myosin heads which agrees well with that previously reported by us for Mg$^{2+}$-ADP binding (21). At 1 mM Mg$^{2+}$-AMP-PNP, the concentration most often used in the literature, there is at least twice as much binding of Mg$^{2+}$-AMP-PNP than Mg$^{2+}$-ADP.

TABLE I

<table>
<thead>
<tr>
<th>Concentration of free AMP-PNP</th>
<th>Impurity</th>
<th>Bound AMP-PNP</th>
<th>Bound AMP-PNP</th>
<th>Myosin heads</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>%</td>
<td>mol</td>
<td>calculated mol</td>
<td></td>
</tr>
<tr>
<td>9.6 x 10^{-6}</td>
<td>10</td>
<td>6.4 x 10^{-4}</td>
<td>0.109</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>9.2 x 10^{-6}</td>
<td>10</td>
<td>6.3 x 10^{-4}</td>
<td>0.111</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>1.4 x 10^{-4}</td>
<td>10</td>
<td>5.6 x 10^{-4}</td>
<td>0.066</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>2.1 x 10^{-4}</td>
<td>10</td>
<td>9.5 x 10^{-4}</td>
<td>0.076</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>3.1 x 10^{-4}</td>
<td>10</td>
<td>1.05 x 10^{-3}</td>
<td>0.085</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>4.3 x 10^{-4}</td>
<td>10</td>
<td>2.8 x 10^{-3}</td>
<td>0.162</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>6.2 x 10^{-4}</td>
<td>10</td>
<td>4.2 x 10^{-3}</td>
<td>0.105</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>9.7 x 10^{-4}</td>
<td>10</td>
<td>5.7 x 10^{-3}</td>
<td>0.095</td>
<td>2.6 x 10^{-7}</td>
</tr>
</tbody>
</table>

Average = 0.09

|          |                  | 2.2 x 10^{-7}         | 2.2 x 10^{-7} |
| 3.0 x 10^{-4} | 4 | 4.9 x 10^{-4} | 0.029    |
| 4.1 x 10^{-4} | 4 | 7.2 x 10^{-4} | 0.030    |
| 6.2 x 10^{-4} | 4 | 1.4 x 10^{-3} | 0.037    |
| 9.2 x 10^{-4} | 4 | 1.1 x 10^{-3} | 0.021    |
| 1.4 x 10^{-3} | 4 | 2.4 x 10^{-3} | 0.030    |

Average = 0.03

*AMP-PNP includes AMP-PNP and any UV-absorbing impurities.

*Measured by isotachophoresis.
AMP-PNP Binding to Myofibrils

Fig. 2. Fraction of myosin heads in rabbit skeletal myofibrils containing bound Mg$^{2+}$-ADP at 0°C in 50% ethylene glycol as a function of free Mg$^{2+}$-ADP concentration. Solid line is the best least-squares fit to the data using the parameters listed in Table II. Dashed line is a linear term representing nonspecific binding needed to fit the data at high Mg$^{2+}$-ADP concentrations. See "Experimental Procedures" for conditions.

binding as that seen in water (Fig. 1). In addition, the Scatchard plot of these data, shown in Fig. 4, reveals a very pronounced curvature both in the presence and absence of Ca$^{2+}$.

A nonlinear, least-squares minimization computer program was used to fit the data in Fig. 3 to a model consisting of a single class of binding sites exhibiting negative cooperativity by using Equation 1,

$$f = \frac{f_C}{K_a + C^n}$$

Where C is the concentration of free Mg$^{2+}$-AMP-PNP, $K_a$ is an apparent binding constant, n is the Hill coefficient, and f is the amount of binding sites containing bound AMP-PNP expressed as a fraction of the calculated amount of myosin heads (f$_0$). The parameter values giving the best fit are shown in Table II and were used to draw the theoretical lines in Figs. 3 and 4. Terms for small amounts of nonspecific binding (less than 3% of f$_0$ at half-saturation) and very tight binding (4.5% of f$_0$) were also included in the fitting, but they could be set to zero without significantly changing the parameter values listed in Table II.

One could have used a model that assumes two classes of binding sites instead of negative cooperativity, but this would have entailed fitting four parameters instead of three. Since both models were found to fit the data equally well, I have chosen to use the simplest model, but there may actually be two pre-existing types of binding sites which bind ADP equally well but not AMP-PNP. Taylor et al. (23), for instance, present evidence that in rigor insect muscle there are two different cross-bridge conformations.

Fig. 3 shows that Mg$^{2+}$-AMP-PNP binding has a pronounced Ca$^{2+}$ sensitivity in 50% ethylene glycol. The fitted parameters in Table II, however, reveal that it is only the affinity that is affected by Ca$^{2+}$ and not the Hill coefficient or the number of binding sites.

**DISCUSSION**

Binding in 50% Ethylene Glycol—Marston and Tregear (18) have shown that with myosin, the major change in going from water to 50% ethylene glycol is to decrease its affinity for actin by a factor of 100 without affecting its affinity for nucleotides. In their studies of insect and rabbit muscle fibers (11, 24, 25), they found that 50% ethylene glycol was just enough to allow 1 mM Mg$^{2+}$-AMP-PNP to eliminate tension and reduce stiffness to the low values seen in muscle fibers relaxed with ATP, implying that under these conditions, the myosin cross-bridges are dissociated from the thin filament.

In this paper, I have shown that Mg$^{2+}$-AMP-PNP binding to myofibrils is sensitive to glycol, whereas Mg$^{2+}$-ADP binding is not. The findings of Marston and Tregear (18) suggest a simple explanation for this difference: Mg$^{2+}$-AMP-PNP binding is thermodynamically coupled to actin-myosin dissociation so that anything which affects the strength of the actin-myosin interaction (i.e. glycol) will also affect the binding of Mg$^{2+}$-AMP-PNP. By this argument, Mg$^{2+}$-ADP is not strongly coupled to dissociation so that glycol has little effect on its binding. This model is summarized in Scheme I.

**Scheme I**

$K_4$ $AM \xleftarrow{+N} AMN$ $K_1$ $K_5$

$M \xleftarrow{+N} MN$

Where A is actin, M is myosin, and N is nucleotide. Since Mg$^{2+}$-AMP-PNP binding causes dissociation, its affinity

**Table II**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Solvent</th>
<th>$K_a$</th>
<th>$K_a^*$</th>
<th>Hill coefficient</th>
<th>Bound nucleotide/myosin head at saturation</th>
<th>Concentration at which there is half-saturation</th>
<th>From Ref. 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$-ADP</td>
<td>Water</td>
<td>120$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$-ADP</td>
<td>50% ethylene glycol</td>
<td>360 ± 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$-AMP-PNP</td>
<td>Water</td>
<td>&gt;1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$-AMP-PNP</td>
<td>50% ethylene glycol</td>
<td>80 ± 14</td>
<td>0.6 ± 0.1</td>
<td></td>
<td>0.8 ± 0.1$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$-AMP-PNP</td>
<td>50% ethylene glycol, no Ca$^{2+}$</td>
<td>22 ± 1</td>
<td>0.60 ± 0.08</td>
<td>0.76 ± 0.05$^d$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Concentration at which there is half-saturation.

$^b$ From Ref. 21.

$^d$ Includes 0.09 from tight binding sites assumed to have the affinity of free myosin subfragment 1 ($K_a = 0.5 \mu M$ from Ref. 22). Does not include nonspecific binding.

$^d$ Includes 0.045 from tight binding sites assumed to have the affinity of free myosin subfragment 1 ($K_a = 0.02 \mu M$ from Ref. 22).
would be equal to $K_dK_5$, whereas Mg$^{2+}$-ADP affinity would be equal to just $K_5$ with little or no contribution from $K_4$. Since Marston and Tregear (18) found that glycol affected $K_4$ but not $K_5$, it would be reasonable to expect that $K_5$ is sensitive to glycol but not $K_4$.

Ca$^{2+}$ Sensitivity—The Ca$^{2+}$ sensitivity of Mg$^{2+}$-AMP-PNP binding seen in Fig. 3 is further evidence of the thermodynamic coupling of Mg$^{2+}$-AMP-PNP binding to actin-myosin dissociation.

Williams and Green (26) have shown that the absence of Ca$^{2+}$ increases the affinity of myosin subfragment 1 for regulated actin by a factor of about 3 in the presence of 1 mM Mg$^{2+}$-AMP-PNP. Bremel and Weber (27) have also shown that Ca$^{2+}$ binding is coupled to dissociation of actin and myosin by ATP in both myofibrils and regulated actomyosin. Thus, Ca$^{2+}$ concentration is another factor which appears to affect primarily the dissociation of actin and myosin rather than the binding of nucleotides themselves. This parallel behavior of Ca$^{2+}$ and glycol is supported by my finding that the Mg$^{2+}$-ADP binding of cardiac myofibrils in aqueous solution is not Ca$^{2+}$-sensitive.$^3$

Biphasic Binding—The Scatchard plot of the Mg$^{2+}$-AMP-PNP binding data in 50% ethylene glycol has a pronounced curvature both with and without Ca$^{2+}$ (Fig. 4) and requires a more complex interpretation than the simple binding seen with Mg$^{2+}$-ADP. Similar biphasic binding of nucleotides to muscle fibers (5) and to myofibrils (28) has been reported in the past and can be explained by either of two basic models: different classes of pre-existing binding sites or negative cooperativity among initially identical binding sites. A negative cooperativity model (Equation 1) was chosen for analysis of the data because it requires the fitting of only three parameters instead of the four required for two classes of sites. A two-class model, however, fits the data equally well, and independent experimentation will be required to make a definitive choice between them. However, the presence of only a single class of Mg$^{2+}$-ADP-binding sites implies that if multiple classes of sites are involved, they do not simply differ in degree of steric hindrance or accessibility of the binding site as a whole.

Taylor et al. (23) have found evidence for two classes of cross-bridges in insect muscle fiber which differ in their attachment to actin filaments. If the only difference between the classes is that one binds actin more weakly than the other, then this difference would be expected to affect the binding of ATP and AMP-PNP, which are strongly coupled to dissociation, much more than ADP, which our studies indicate is not strongly coupled to dissociation.

Aqueous Solution Binding—The binding of Mg$^{2+}$-AMP-PNP to skeletal myofibrils in aqueous solution was found to be quite weak ($K_d > 1000 \mu M$). In the literature, however, equilibrium binding experiments have been reported to give dissociation constants as low as 30 $\mu M$ for whole fibers (13, 29), while a kinetically determined $K_d$ for fibers has been reported by Pate and Cooke (30) to be 3000 $\mu M$. A similar situation exists in the literature for Mg$^{2+}$-AMP-PNP binding to skeletal actin-myosin subfragment 1 complex: a discrepancy between equilibrium and kinetically derived binding constants (22, 31) has been cited as evidence for more than one conformation of the myosin head (33), whereas another laboratory has recently revised its previously reported equilibrium binding constant of Mg$^{2+}$-AMP-PNP to actin-myosin subfragment 1 complex from 200 to 2000 $\mu M$ (32).

Future efforts will be directed toward clarifying the aqueous binding properties of Mg$^{2+}$-AMP-PNP to both myofibrils and actin-myosin subfragment 1 complex in order to help determine the validity of the Mg$^{2+}$-AMP-PNP-actin-myosin ternary complex as a model for the second bound form of the myosin cross-bridge.

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REFERENCES

AMP-PNP Binding to Myofibrils