Cold-sensitive Mutants G680V and G691C of Dictyostelium Myosin II Confer Dramatically Different Biochemical Defects

(Received for publication, June 11, 1997, and in revised form, August 27, 1997)

Bruce Patterson‡§, Kathleen M. Ruppel‡, Yuan Wu§, and James A. Spudich¶

From the Departments of Molecular and Cell Biology, University of Arizona, Tucson, Arizona 85721 and the Departments of Biochemistry and Cell Biology, Stanford University, Stanford, California 94305-5307

Cold-sensitive myosin mutants represent powerful tools for dissecting discrete deficiencies in myosin function. Biochemical characterization of two such mutants, G680V and G691C, has allowed us to identify separate facets of myosin motor function perturbed by each alteration. Compared with wild type, the G680V myosin exhibits a substantially enhanced affinity for several nucleotides, decreased ATPase activity, and overoccupancy or creation of a novel strongly actin-binding state. The properties of the novel strong binding state are consistent with a partial arrest or pausing at the onset of the mechanical stroke. The G691C mutant, on the other hand, exhibits an elevated basal ATPase indicative of premature phosphate release. By releasing phosphate without a requirement for actin binding, the G691C can bypass the part of the cycle involving the mechanical stroke. The two mutants, despite having alterations in glycine residues separated by only 11 residues, have dramatically different consequences on the mechanochemical cycle.

The study of the family of molecular motors recently has been invigorated by the availability of crystal structures of both the myosin (1–5) and kinesin family members (6, 7). This information, combined with a wealth of biochemical characterization of these motors, provides a platform on which to build a complete understanding of the mechanics of these pivotal cellular components. In a complementary approach, we have been generating conditionally functional (cold-sensitive) mutants of myosin II in the Dictyostelium system (8, 9). In our isolation of cold-sensitive myosin mutants, we uncovered two mutations in a biochemically renowned region of the protein, a helical element that houses two highly reactive cysteines in rabbit myosin.

This region of myosin was first characterized because of the chemical accessibility of two cysteines contained within it (positions 697 (SH2) and 707 (SH1) in rabbit myosin). The crystal structure of chicken pectoralis muscle S1 displays these cysteines in a bent α-helix, separated from one another by 18 Å and facing opposite sides of the molecule (2). However, cross-linking experiments using rabbit skeletal muscle myosin demonstrat that during the stroking cycle, the two approach more closely than in the absence of nucleotide (10) and can be directly joined by a disulfide linkage, indicative of a separation of only about 3 Å and a substantially altered geometry (11). The conformational changes are not purely local, in that either cysteine can cross-link with residues in other parts of the structure (for example, SH1-Cys522 and SH2-Cys546 in rabbit (12, 13)) during the cycle. Chemical modification studies have demonstrated that addition of bulky hydrophobic groups to the cysteines dramatically changes the properties of the myosin molecule, particularly with regard to the promiscuity and activity of its ATPase. The physiological substrate of myosin is MgATP which supports only low-level ATPase activity in the absence of actin. However, if provided with CaATP or KATP, rabbit myosin shows dramatically enhanced ATPase activity in the absence of actin. Modification of either thiol further activates the calcium ATPase, while preventing the potassium ATPase (14). Furthermore, cross-linking the reactive thiols using a variety of linking agents results in a molecule lacking measurable ATPase activity and holding a “trapped” nucleotide in its catalytic site. Thus the “thiol region” of the molecule is not only mobile, it markedly influences events at the active site.

In Dictyostelium myosin II, one of the thiol positions (SH1) is occupied by a threonine residue (Thr680). Nonetheless sequence conservation in this region allows unambiguous alignment of the myosin sequences from chicken and rabbit muscle with Dictyostelium myosin II (Fig. 1). The bend in the helix in which the two cysteines lie occurs at an absolutely conserved glycine residue, and the C terminus of the helix is demarcated by a second absolutely conserved glycine residue. We recovered alterations at both the bend glycine (position Gly680 in Dictyostelium myosin II) and the C-terminal glycine (position Gly691 in Dictyostelium) in a screen for cold-sensitive myosin mutants arising from random mutagenesis (8, 9). Replacement of these glycines by bulkier residues has previously been suggested by Huston et al. (11). Cold-sensitivity resulting from alteration of either of these glycines suggests the exciting possibility that these mutations block transitions between conformations occurring during the motor cycle, trapping these conformations in the absence of sufficient thermal energy. We have undertaken a thorough biochemical characterization of these mutants, as well as limited characterization of glycine-to-alanine changes at each position, to uncover information about the conformations and roles of this region in the cycle.

One of the mutations at a position corresponding to Gly680 (Gly680 in the rat skeletal muscle myosin used for the study) has also been characterized by Kinose et al. (15) through site-directed mutagenesis (15). Their analysis of motility and actin-activated ATPase led them to focus on alterations of product release properties of the motor. Our findings suggest that this is indeed one facet of the aberrant behaviors of Gly680 alter-
Biochemical Analysis of Cold-sensitive Myosin II Mutants

EXPERIMENTAL PROCEDURES

Chemicals—ATP and ADP (disodium salt, approximately 99% pure according to the manufacturer) were purchased from Sigma. Restriction enzymes and T4 DNA ligase were from New England Biolabs. ATPS1 (tetralithium salt, >85% pure according to the manufacturer) was purchased from CalBiochem. All other reagents were from Sigma unless otherwise noted. Alkaline phosphatase was purchased from Boehringer Mannheim.

Preparation of full-length myosins was performed as described (16). Briefly, Dictyostelium cytoskeletons were prepared by mechanical lysis in an EDTA-containing low-salt buffer, followed by extraction of myosin with a MgATP-containing high-salt buffer. This was followed by two rounds of salt-dependent assembly-disassembly of myosin filaments accompanied by low-speed spins to remove contaminating material or to recover myosin filaments, as appropriate. The purified proteins were treated with Dictyostelium myosin light chain kinase as described previously (17).

Preparation of Myosin S1—Myosin S1 was synthesized from a truncated myosin gene, encoding the normal or mutant Dictyostelium sequence up to amino acid Val63, which was followed by sequences encoding Pro-His-His-His-His-His-His-STOP. This myosin S1 was prepared by a modification of the procedure of Manstein et al. (18). Approximately 100 million cells were recovered from surfaces and washed in 15 ml of ice-cold Bonner’s solution (10 mM NaCl, 10 mM KCl, 2 mM CaCl2), followed by addition of 20 mM Na3, for 10 min. Cells were washed with 1 ml of wash buffer (50 mM Tris-HCl, pH 8.0, at 4 °C, 25 mM EGTA, 2 mM EDTA, 5 mM benzamidine, and 2 mM 2-mercaptoethanol) containing 10 mM ATP and ADP (disodium salt, approximately 99% pure). Cytoskeletons were resuspended in 50 mM Tris-HCl, pH 8.0 at 4 °C, and washed once with 500 mM Na3, for 10 min. The supernatants were discarded. Pellets were resuspended in the Wash Buffer Plus and run on a SDS-PAGE gel. The myosin S1 bands were stained by Coomassie Blue and scanned on a transmissive scanner. Quantification of relative S1 amount in the different bands on the same gel was done by the NIH Image software. 

Plasmid Constructions—All mutants were created by site-directed mutagenesis of M13.Myo.Cend using oligonucleotides 860.mute (5′-TTGGCAATG(C/T)TGTCCTG), 691A.mute (TCGTAAAGCTTTCCAA), and 691C.mute (TCGTAAATGTTTCCAA), and 691C.mute (TCGTAAATGTTTCCAA). Double-stranded phage DNA was prepared and the BglII-NcoI fragment containing the mutations was used to replace the BgII-NcoI fragment of PBG.Myo.SXX. 

RESULTS

In Vitro Motility—We first sought to reproduce in vitro the observed in vivo failures of the G680V and G691C myosins by characterizing their ability to support actin filament sliding in vitro. Under standard conditions, both mutants supported poor movement (Fig. 2), consistent with their myosin-deficient phenotypes in vivo. Filament sliding is on the order of 10% the wild type rate for each mutant. To ascertain whether either of the mutants could have a braking effect on motility supported by

1 The abbreviation used is: ATP, adenosine 5′-O-(thiotriphosphate).

2 T. Q.-P. Uyeda, unpublished data.
TABLE I

**ATPase activities of mutant and wild type myosins**

ATPase activity for basal ATPase, actin-activated ATPase, and high-salt Ca\(^{2+}\)-ATPase are shown for wild type and mutant myosins at 13, 21, and 30 °C.  

<table>
<thead>
<tr>
<th>Temperature</th>
<th>WT</th>
<th>G680A</th>
<th>G680V</th>
<th>G691A</th>
<th>G691C</th>
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</thead>
<tbody>
<tr>
<td>Basal</td>
<td>13</td>
<td>18</td>
<td>5.1</td>
<td>3.6</td>
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<tr>
<td></td>
<td>21</td>
<td>ND*</td>
<td>16</td>
<td>59</td>
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</tr>
<tr>
<td></td>
<td>30</td>
<td>ND</td>
<td>26</td>
<td>19</td>
<td>184</td>
</tr>
<tr>
<td>Actin-activated</td>
<td>13</td>
<td>132</td>
<td>48</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td></td>
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<td>30</td>
<td>414</td>
<td>400</td>
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<td>579</td>
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<tr>
<td>High-salt/Ca(^{2+})</td>
<td>13</td>
<td>181</td>
<td>45</td>
<td>5.6</td>
<td>236</td>
</tr>
<tr>
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<td>190</td>
<td>18</td>
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</tr>
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<td></td>
<td>30</td>
<td>472</td>
<td>159</td>
<td>19</td>
<td>653</td>
</tr>
</tbody>
</table>

* ND, none detected.

wild-type myosin, we performed mixing experiments in which the normal amount of wild type filaments were first mixed with 10% of their number of mutant filaments. The mixture was then applied to the coverslip and assayed under standard conditions. The mutants demonstrated dramatically different properties: while the mixture containing 10% G691C mutant myosin moved similarly to a pure wild-type population, the mixture containing 10% G680V strongly resembled a pure mutant population. The simplest interpretation of this result is that the G680V mutant has created or elongated a strongly bound state, such that a minority population of mutant molecules can restrict movements generated by wild type myosin acting on the same filament. The G691C mutant, on the other hand, did not demonstrate any deleterious effect on wild type motility, suggesting that it does not over-occupy a strongly bound state.

**ATPase Activity**—We next characterized the ATPase activities of each mutant. We examined: 1) basal Mg\(^{2+}\)-ATPase, which measures hydrolysis of ATP in the absence of activation by actin (note that the rate-limiting step in this process for wild-type myosins is actually P\(_i\) release rather than hydrolysis per se); 2) actin-activated ATPase; and 3) high-salt Ca\(^{2+}\)-ATPase, a non-physiological activity that entails bypass of the actin requirement for product release. For most myosin IIs, including *Dictyostelium*‘s, the latter ATPase has a rate comparable to the actin-activated ATPase. For these studies, we employed full-length myosins, and characterized not only the G680V and G691C myosins, but also structurally milder mutations at each position created by site-directed mutagenesis. The milder forms were G680A and G691A, created on the rationale that the small, non-hydrophobic alanine would be a more acceptable substitute for glycine than cysteine or valine. Each assay was performed at 13, 21, and 30 °C to determine if we could correlate any *in vitro* properties with the *in vivo* cold-sensitivity of the mutants as assessed by the azide assay (9).

The most marked alteration caused by position 691 changes was in the basal ATPase results. While wild-type ATPase activity is just detectable in the assay, both G691A and G691C myosins have dramatically enhanced activity (Table I and Fig. 3). This enhancement is present at all temperatures tested, and increases with increasing temperature. At each temperature, the ATPase rate of the G691C mutant is greater than the G691A mutant, in concordance with the relative severity of the two mutants *in vivo* and the anticipated structural alterations. Thus both position 691 mutants “unlock” the basal ATPase. Since this reaction is limited by product release, specifically release of P\(_i\), we conclude that substitution of bulkier residues for Gly\(^{691}\) enhances the release or escape of inorganic phosphate in the absence of actin.

Addition of actin to wild-type myosin measurably enhances its ATPase activity (Table I and Fig. 3) yielding a roughly 10-fold increase compared with basal ATPase at each temperature. The position 691 mutants actually have lower actin-activated ATPases than wild type at 13 and 21 °C, but each has greater actin-activated ATPase at 30 °C. Even more striking is the comparison of the actin-activated ATPase to the basal ATPase: at 13 °C both mutants show greater or equal ATPase activity in the absence of actin than in its presence (Table I); in other words, there is no evidence that actin plays a role in stimulating the ATPase. This situation is ameliorated at 21 °C: the G691A actin-activated ATPase is approximately 3-fold greater than its basal ATPase, while the G691C actin-activated ATPase reaches 1.5 times its basal level at the same temperature. Both mutants also show activation induced by actin at 30 °C: 3-fold for G691A and 2.2-fold for G691C. These data

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3 B. Patterson, unpublished observations.
probably reveal the phenomenon underlying the cold-sensitivity of the mutants in vivo: obviation of the actin binding requirement for release of P<sub>i</sub> at low temperatures. The Ca<sup>2+</sup>-ATPases for position 691 mutants are greater than wild-type, but this relative increase is not as dramatic as that observed for their basal ATPases. As with wild-type, the Ca<sup>2+</sup>-ATPases are higher than the actin-activated ATPases at each temperature, but a strict correlation cannot be drawn between the extent of the high-salt Ca<sup>2+</sup>-ATPases and the failures of the mutants in vivo.

The G680A and G680V mutants behave very differently from those at position 691 (Table I and Fig. 3). If anything, the basal ATPases of these mutants are lower than those of wild-type. The actin-activated ATPases of the two mutants are notable: for G680A myosin, the 30 °C actin-activated ATPase is comparable to that of wild-type, whereas at 21 °C the value for G680A myosin is half that of wild-type, and at 13 °C it is substantially less than half. The basic pattern is similar for G680V myosin, but the deficiency is more pronounced: while at 30 °C the activity is roughly half that of wild-type, at 21 and 13 °C, the deficit is greater still: actin-activated ATPases are at 36 and 19% of wild-type levels, respectively. It is interesting to note, however, that the extent of activation conferred by addition of actin to the basal ATPase is as good or better for the two mutants in comparison to wild-type (i.e. the activity is stimulated ~10-fold in each case). Finally, the high-salt Ca<sup>2+</sup>-ATPases reveal a striking difference between wild-type and position 680-altered mutants. While for wild-type, these conditions give rise to greater activity than actin-activation, for both position 680 mutants, this is not the case. While G680A myosin shows moderate ATPase activity in the presence of high-salt and Ca<sup>2+</sup>-ATP, that of G680V myosin is virtually undetectable at all three temperatures. This effect appears to be specifically due to a failure of Ca<sup>2+</sup> to induce the ATPase, since addition of high-salt in amounts comparable to those found in the Ca<sup>2+</sup>-assay to the basal (Mg<sup>2+</sup>) ATPase elevates this ATPase rather than inhibiting it. Thus for the G680 mutants, we have a more stringent requirement for actin to complete the ATPase cycle and a failure to be induced by the biologically unlikely substrate Ca<sup>2+</sup>-ATP.

Actin Co-sedimentation—To further delineate the biochemical deficiency underlying the inadequacies of the G680V mutant, we investigated the actin binding properties of the mutant expressed as an S1 (amino acids 1–865 followed by a proline and a 6-histidine tag) and characterized at the non-permissive temperature (13 °C) under a variety of conditions. For wild-type, we reasoned that failure of the mutant S1 to bind ATP was unlikely to be the cause of the observed failure to release from actin. We confirmed this by demonstrating that the K<sub>m</sub> of the G680V-S1 for ATP as measured by actin-activated ATPase is approximately 120 μM, comparable to that of wild-type, for which we calculate a K<sub>m</sub> of 97 μM.

Since the G680V-S1 bound ATP with high affinity and progressed through the cycle in the presence of actin (as evidenced by ATPase activity), we tested the hypothesis that the strong-binding defect of the mutant lay in an inability to release ADP by ATPase activity), we tested the hypothesis that the strong-binding defect of the mutant lay in an inability to release ADP at the end of the cycle, i.e. the mutant had an enhanced affinity for ADP. We investigated this possibility by taking advantage of the demonstrated affinity of G680V-S1 for ATP<sub>γ</sub>S. Since ATP-γ-S binds with high affinity and releases G680V-S1 from actin even at low salt, we assayed for the ability of ADP to compete for G680V-S1 heads as measured by actin binding in the presence of ATP<sub>γ</sub>S. We determined the ratio of ADP/ATP<sub>γ</sub>S at which half the G680V-S1 was bound to actin. This experiment was carried out in the presence of 100 μM ATP<sub>γ</sub>S, a concentration sufficient to almost quantitatively release G680V from actin filaments in the absence of ADP. As shown in Fig. 6, ADP is able to compete successfully with ATP<sub>γ</sub>S for the binding site of G680V at a ratio of 10:1, comparable to that of wild-type. Thus, since the relative affinities of the two mole-

4 Y. Wu and B. Patterson, unpublished observations.

5 B. Patterson and Y. Wu, unpublished data.
of G680V-S1 from actin in the presence of 250 mM KCl, denucleotide-free S1 (23). We therefore analyzed the actin binding rabbit skeletal muscle S1-ADP complex compared with nucleotide-free S1. The greater salt-sensitivity of actin binding displayed by the mutant in the presence of ATP. We sought to take advantage of the exaggerated strong binding to actin shown by the G680V mutant indeed has an enhanced affinity for ADP as well.

We attempted to correlate this increased affinity for ADP with the exaggerated strong binding to actin shown by the mutant in the presence of ATP. We sought to take advantage of the greater salt-sensitivity of actin binding displayed by the rabbit skeletal muscle S1-ADP complex compared with nucleotide-free S1 (23). We therefore analyzed the actin binding behavior of G680V-S1 in the presence of 250 mM KCl at 13 °C. We characterized the amount of G680V-S1 released from actin in the absence of nucleotide, in the presence of 1 mM ADP, and in the presence of increasing amounts of ATP. Under these high-salt conditions we were able to readily discern an effect of ATP on G680V-S1 affinity for actin. Release of significant amounts of G680V-S1 occurred even at the lowest concentration of ATP (3 μM) (Fig. 5). ~75% of the G680V-S1 is released from actin by an ATP concentration of 300 μM, compared with only ~20% release effected by this concentration of ATP in the absence of KCl. This release is demonstrating salt sensitivity of an intermediate in the ATPase cycle as it is observed only in the presence of ATP and not in the absence of nucleotide or in the absence of ATP or ADP. However, despite the rationale that led to the experiment, we find that ATP does not enhance release of G680V-S1 from actin in the presence of 250 mM KCl, demonstrating that the release we are observing in the presence of ATP must be occurring from a complex different from that which can be achieved solely by introduction of ADP.

**DISCUSSION**

Gly860 Mutants Increase Affinity for Nucleotide and Reveal a Novel State—The G680A and G680V mutant myosins exhibit several aberrant properties. The most dramatic finding is derived from the in vitro "dominance" of G680V myosins poor motility even when mixed with a 10-fold excess of wild-type. This braking ability indicates abnormally long occupancy of a strongly bound state. Such a state can arise from at least four alterations: 1) an impaired ability to bind nucleotide, stalling the molecule in the tight-binding "rigor" state; 2) an increase in the affinity of myosin for actin during the weakly bound (ATP and ADP-P states); 3) inability to release ADP with the same kinetics as wild-type; and 4) extension of a normally short-lived strong-binding state or creation of a novel one, resulting in abnormal continuance of a strong-binding myosin-product state or states. The first possibility, inability to bind nucleotide is ruled out since the $K_m$ for ATP that we measure for the G680V mutant is comparable to that of wild-type. Notably, the affinity of myosin for ATP$\gamma$S in the presence of actin is dramatically greater than that of wild-type, as measured by ability of ATP$\gamma$S to release myosin from actin filaments. The latter experiment also addresses possibility number 2, enhanced "weak" binding. Due to its low rate of hydrolysis, ATP$\gamma$S effectively arrests myosin in a state similar to the ATP-bound state. The behavior of G680V-S1 in the presence of sufficient quantities of ATP$\gamma$S mimics wild-type, in that G680V-S1 does not co-sediment with actin in the presence of ATP$\gamma$S. Thus we do not detect any evidence of strengthened weak binding states for G680V-S1.

The third possibility, inability of the G680V mutant to efficiently release ADP, appears to be occurring. This possibility is consistent with, although not dictated by, the demonstrated greater affinity of G680V-S1 for ATP$\gamma$S. We have taken advantage of the affinity of G680V-S1 for ATP$\gamma$S to indirectly demonstrate high affinity for ADP: a similar ratio of ADP is required to compete with ATP$\gamma$S for binding to G680V-S1 as is required to compete for binding of wild type. Thus the relative affinities of ADP and ATP$\gamma$S are the same for mutant and wild-type, and the mutant affinity for ADP must also be 40-fold higher than that of wild type. Second, we have demonstrated that ADP interferes with the actin-activated ATPase of the mutant at much lower levels than are required for interference with the wild-type actin-activated ATPase. Thus overzealous affinity for nucleotide, as it manifests at the end of the cycle with ADP, potentially contributes to the poor motility of the G680V mutant and to its ability to interfere with motility of wild-type myosin in mixing experiments.

The fourth possibility, extension of a novel or normally short-lived strong-binding state, also appears to be the case. The G680V mutant S1 clearly over-occupies a strongly bound state during the ATPase cycle, in that it co-sediments with actin even at ATP levels well above $K_m$ (Fig. 5). Under similar conditions, the majority of wild-type S1 is released, consistent with its spending only a fraction of its ATPase cycle in strongly bound states. Our data further suggest that this strongly bound state does not correspond to canonical rigor or ADP-bound states. Co-sedimentation of G680V-S1 with actin in the presence of ATP is salt-sensitive in a manner not characteristic of the ADP-bound state. In the presence of 250 mM KCl, 75% of G680V-S1 is released from actin by 100 μM ATP, while only ~20% is released in the absence of KCl. Thus at steady state,

![FIG. 5. Cosedimentation of G680V and wild type S1s in the presence of ATP with and without 250 mM KCl. The amount of S1 cosedimenting with actin (expressed as a percentage of amount cosedimenting in the absence of added nucleotide) under varying conditions of ADP or ATP concentration and added in the presence or absence of 250 mM KCl.](image1)

![FIG. 6. Competition of ADP with ATP$\gamma$S for binding to S1 in the presence of actin. The ability of ADP to compete with ATP$\gamma$S, as measured by binding to actin (a property of ADP-bound wild type S1 or G680V-S1, but not of either complex with ATP$\gamma$S bound). Comparisons are to amount of S1 released by 10 mM ATP$\gamma$S in the absence of added ADP.](image2)

6 B. Patterson and Y. Wu, unpublished results.
rate-limiting step, which for this reaction is Pi release. Com-
ATPase rate demonstrates that the mutant must speed up a
behavior compared with wild-type. The accelerated basal
the presence of actin, whereas the normal coupling is merely
ductive interaction with actin, but that the release of phos-
consistent with a model in which the G680V-S1 initiates pro-
states of actin binding might be anticipated. Our data are
lieved to be closely juxtaposed to the onset of tight binding to
phosphate release. This point in the biochemical cycle is be-
rate-limiting step is phosphate release, and the behavior of the
depresses the ATPase rate. Certainly for the basal ATPase, the
data (Fig. 3). Essentially in all cases, mutation at position G680
cause of cold-sensitivity
temperatures. These data are consistent with the underlying
activity brought about by the presence of actin, the G691C
formative: while the wild-type exhibits substantial increases in
activity brought about by the presence of actin, the G691C
reinforces this view of the G691C as a promiscuous ATPase: the

**Alteration of Position 691 Results in Promiscuous Release of**
P<sub>1</sub>—The G691C mutant also has readily identifiable alterations in
behavior compared with wild-type. The accelerated basal
ATPase rate demonstrates that the mutant must speed up a
rate-limiting step, which for this reaction is P<sub>1</sub> release. Com-
paring the basal ATPase to the actin-activated ATPase is infor-
mentative: while the wild-type exhibits substantial increases in
activity brought about by the presence of actin, the G691C
mutant shows little if any enhancement, especially at the lower
truthes. These data are consistent with the underlying
cause of cold-sensitivity in vivo being a failure to efficiently
couple product release (and by implication, the transduction of the
ATP hydrolysis energy into a mechanical stroke) with actin
binding. At the low temperature, P<sub>1</sub> release occurs regardless of the
presence of actin, whereas the normal coupling is merely
inefficient at the permissive temperature. The Ca<sup>2+</sup>-ATPase
reinforces this view of the G691C as a promiscuous ATPase: the
mutant’s activity for this aberrant substrate is even higher than
that of wild type. Thus again we can explain the biological
ineffectiveness of the mutant: by releasing P<sub>1</sub> without “waiting”
to interact with actin, the mutant is able to complete the
ATPase cycle without performing useful work.

**Chemical Nature of Changes**—The fact that both the cold-
sensitive mutants we recovered in this region are glycine alter-
tations was one of the factors that led us to analyze them. The
possibility that the mutant phenotypes arise from substi-
tution of a flexible residue with more constrained ones is con-
consistent with our goal of isolating myosins restricted in their
ability to undergo critical conformational changes (8). The be-
behavior of the glycine → alanine changes (G680A and G691A)
are consistent with this interpretation: in each case, the com-
paratively small alanine side chain (in contrast to valine or
cysteine) confers a defect essentially identical in direction to the
larger alteration recovered in the screen, but markedly
less in degree. Thus G680A myosin has almost normal actin-
activated ATPase activity at 30 °C and detectable Ca<sup>2+</sup>-ATPase at all temperatures, while the G680V mutant myosin has only ~50% of the wild type actin-activated ATPase at 30 °C, proportionally even less at the lower temperatures, and barely detectable Ca<sup>2+</sup>-ATPase at any temperature. The G691A mutation similarly has less elevated basal ATPases at all temperatures than G691C, and shows greater actin-activation (as compared with basal ATPase) than G691C at 21 and 30 °C. These findings are consistent with the interpretation that the mutants make adoption of certain states in the motor cycle more favorable or more stable than for wild type. Crys-
tallographic analysis of these mutations may therefore yield
fresh insights into the different structural states of the myosin
cycle.

**Acknowledgments**—We gratefully acknowledge Emil Reisler, Taro
Uyeda, Kim Giese, and Roy Parker for enlightening discussions.

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doi: 10.1074/jbc.272.44.27612

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