Engineering of the Myosin-Iβ Nucleotide-binding Pocket to Create Selective Sensitivity to N⁶-modified ADP Analogs*

(Received for publication, April 30, 1999, and in revised form, July 21, 1999)

Peter G. Gillespie‡‡§§, Susan K. H. Gillespie‡§, John A. Mercer**, Kavita Shah‡‡, and Kevan M. Shokat‡‡ §§

From the Departments of Physiology and Neuroscience, The Johns Hopkins University, Baltimore, Maryland 21205, ‡‡McLaughlin Research Institute, Great Falls, Montana 59405, and the §§Departments of Chemistry and Molecular Biology, Princeton University, Princeton, New Jersey 08544

Distinguishing the cellular functions carried out by enzymes of highly similar structure would be simplified by the availability of isozyme-selective inhibitors. To determine roles played by individual members of the large myosin superfamily, we designed a mutation in myosin's nucleotide-binding pocket that permits binding of adenine nucleotides modified with bulky N⁶ substituents. Introduction of this mutation, Y61G in rat myosin-Iβ, did not alter the enzyme's affinity for ATP or actin and actually increased its ATPase activity and actin-translocation rate. We also synthesized several N⁶-modified ADP analogs that should bind to and inhibit mutant, but not wild-type, myosin molecules. Several of these N⁶-modified ADP analogs were more than 40-fold more potent at inhibiting ATP hydrolysis by Y61G than wild-type myosin-Iβ; in doing so, these analogs locked Y61G myosin-Iβ tightly to actin. N⁶-(2-methylbutyl) ADP abolished actin filament motility mediated by Y61G, but not wild-type, myosin-Iβ. Furthermore, a small fraction of inhibited Y61G molecules was sufficient to block filament motility mediated by mixtures of wild-type and Y61G myosin-Iβ. Introduction of Y61G myosin-Iβ molecules into a cell should permit selective inhibition by N⁶-modified ADP analogs of cellular processes dependent on myosin-Iβ.

Myosin molecules carry out mechanical work within cells, hydrolyzing ATP to produce force along actin filaments (1, 2). The myosin superfamily contains at least 15 major classes, eight or more of which are found in vertebrates. Because multiple myosin isozymes are found within each class, the superfamily is large; for example, the murine genome has more than 30 myosin genes (3, 4). Myosin molecules share a common three-domain structure: an actin- and ATP-binding head that tightly can be changed from guanine nucleotide- to xanthine nucleotide-dependent by changing a conserved Asp (in the motif NKX) to Asn (17–24). Dependence on xanthine triphosphates following introduction of mutated GTPases signals the participation of the mutated protein (21). In a second example, nucleotide-binding pockets of protein-tyrosine kinases can be altered to accept certain N⁶-substituted adenosine triphosphates, which otherwise did not serve as substrates for known kinases (25, 26). The mutated kinases were also sensitive to certain pyrazolo[3,4-d]pyrimidines, membrane-permeant inhibitors that could revert morphological changes associated with kinase-mediated cell transformation (27).

We chose instead to design a mutation that would render a myosin isozyme sensitive to a nucleotide analog that otherwise did not bind native myosin molecules. Once these mutant myosin molecules have been introduced into a cell, processes dependent on that isozyme should become sensitive to the inhibitor. This strategy uses as its inspiration other examples of modification of enzymatic specificity by protein engineering. For instance, the substrate specificity of the GTPase superfamily can be changed from guanine nucleotide- to xanthine nucleotide-dependent by changing a conserved Asp (in the motif NXXD) to Asn (17–24). Dependence on xanthine triphosphates following introduction of mutated GTPases signals the participation of the mutated protein (21). In a second example, nucleotide-binding pockets of protein-tyrosine kinases can be altered to accept certain N⁶-substituted adenosine triphosphates, which otherwise did not serve as substrates for known kinases (25, 26). The mutated kinases were also sensitive to certain pyrazolo[3,4-d]pyrimidines, membrane-permeant inhibitors that could revert morphological changes associated with kinase-mediated cell transformation (27).

We have focused our attention on myosin-Iβ, an isozyme hypothesized to mediate adaptation of auditory and vestibular mechanical transduction (28). We have designed a missense mutant of rat myosin-Iβ, replacing tyrosine-61 with glycine...
(Y61G), which has little effect on ATP hydrolytic activity yet renders the mutant sensitive to N\textsuperscript{0}-modified adenosine diphosphates. These analogs inhibit ATP hydrolysis by preventing myosin dissociation from actin, inducing a tightly bound state.

**EXPERIMENTAL PROCEDURES**

**Materials**—Life Technologies, Inc. was the source of Grace’s medium, lactalbumin hydrolysate, yeastolate, Fluronic F-68, gentamicin, and fetal calf serum. The pBlueBacHis2B transfer vector, linearized DNA from *Autographa californica* nuclear polyhedrosis virus, cationic liposomes, and anti-DLYDDDDK antibodies were purchased from Invitrogen (San Diego, CA). Restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs (Beverley, MA). Reagents for unique-site elimination, Superdex 200 columns, phenyl-Sepharose, DEAE-Sephadex, and 1\textsuperscript{y}-3\textsuperscript{y}PIATP were from Amersham Pharmacia Biotech. N\textsuperscript{0}\textsuperscript{-}charged nitrotriacetic acid-agarose (Ni\textsuperscript{2+}-NTA)\textsuperscript{1} was obtained from Qiagen (Valencia, CA). Frozen bovine brains and rabbit muscle acetone powder (special order) were purchased from Pel-Freez (Rogers, AR). Microtiter plates were either Immulon from Dynex (West Sussex, UK), or high binding plates from Xenopore (Hawthorne, NJ). All SDS-polyacrylamide gel electrophoresis, dithiothreitol, Tween 20 (50 h), and Bio-Rad. Secondary antibodies were from Southern Biotechnology Associates (Birmingham, AL), whereas bichronic acidic protein assay reagents and p-nitrophenol phosphate were from Pierce. ATP, ADP, catalase, glucose oxidase, and protein A were from Sigma. Adenosine 5\textsuperscript{-}O-(2-thiodiphosphate) (ADP\textsuperscript{S}) and bovine serum albumin were purchased from Calbiochem, whereas rhodamine-phalloidin was from Molecular Probes (Eugene, OR). Nicotinamide was from Sigma alcohol was from Ernest Fullam (Latham, NY). Insight II (version 4.0.0), used to prepare the structure in Fig. 1A, was obtained from Molecular Simulations (San Diego, CA).

**Synthesis of ATP Analogs—**Analogs 1-6 (see Fig. 5B) were synthesized as described previously (25); 6-chloropurine riboside (Aldrich) was refurred with aniline, benzylamine, 2-phenethylamine, 3-methylbenzylamine, 1-methylbutylamine, or 2-methylbutylamine, respectively, in ethanol overnight (25). Trifluorophosphate synthesis was carried out as described previously (25).

N\textsuperscript{0}-modified nucleoside diphosphates were isolated as a by-product (about 20% of total) of the trifluorophosphate synthesis. Nucleotides were purified on DEAE-Sephadex (A-25) using a linear gradient of 0.1–1.0 M triethylammonium bicarbonate at pH 7.5. These compounds were characterized by \textsuperscript{1}H NMR and mass spectral analysis. Nucleotides were also characterized by high pressure liquid chromatography on a strong anion-exchange column (Rainin SAX-83-E03-ETI), using a linear gradient of 5–750 mM ammonium phosphate, pH 3.9, for 10 min, followed by isocratic elution at 750 mM ammonium phosphate for 10 min. Typical retention time difference between diphosphates and triphosphates was about 2 min.

**Construction of Myosin-I\textsuperscript{B} Baculoviruses**—Using the polymerase chain reaction, we modified the pBlueBacHis2B baculovirus transfer vector by adding an NcoI site immediately following the enterokinase cleavage site. We also removed an NcoI site in the original multiple cloning site by KpnI digestion, removal of 3\textsuperscript{y} overlaps with T4 DNA polymerase, and deoxyribonucleotides, and religation; the modified vector was termed pBlueBacHis2B-Nco. The plasmid C myr2 tag pCMV5 (30), containing the cDNA sequence for rat myosin-I\textsuperscript{B} with a COOH-terminal myc epitope tag (courtesy of Dr. Martin Bähler), was digested with NcoI and BamHI; after purification, myosin-I\textsuperscript{B} cDNA was ligated to NcoI- and BamHI-digested pBlueBacHis2B-Nco to generate the plasmid pBBHis2B-rmyoI. Following sequencing was carried out to ensure the fidelity of the cloning junctions. When expressed, the recombinant myosin-I\textsuperscript{B} contained an extra 34 amino acids at its NH\textsubscript{2} terminus, including a hexahistidine tag, an antibody epitope (DLYDDDDK), and an entry/\n
---

\textsuperscript{1} The abbreviations used are: Ni\textsuperscript{2+}-NTA, nickel-nitrilotriacetic acid; ADP\textsuperscript{S}, adenosine 5\textsuperscript{-}O-(2-thiodiphosphate); PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

---

\textsuperscript{2} S. Jean and P. G. Gillespie, unpublished data.
ured in 10 μl total volume; after mixing components in 1.7-ml siliconized microfuge tubes and initiating the reaction with γ-32P-ATP or a mixture of actin and myosin, tubes were centrifuged for ~5 s. After incubation at 37 °C for 10–40 min, reactions were terminated with silicotungstic acid and sulfuric acid and γ-32P-P, was recovered using isobutanol and benzene (1:1) and ammonium molybdate as described (40). To account for the intrinsic ATPase activity of actin and its inhibition by adenine nucleotides, we always included in our assays control samples lacking myosin-β but including actin and appropriate nucleotides.

Data were plotted as the mean ± S.D. of 2–5 (usually 3) samples. Inhibition data in Figs. 4, 6, and 7 were fit with:

\[
\text{Velocity (% of control)} = \frac{[N]}{[N] + IC_{50}}
\]

(1) where \([N]\) is the concentration of the nucleotide analog and \(IC_{50}\) is the concentration yielding 50% inhibition. \(IC_{50}\) values were occasionally extrapolated from data sets showing very little inhibition; confidence in these \(IC_{50}\) values was therefore poor (e.g. inhibition of wild-type myosin-β by some analogs). Binding data in Fig. 7 were plotted as described in the legend.

**Actin-Myosin-β Binding Assay**—The standard buffer was ATPase assay solution. Myosin (0.02–0.1 μM) and actin (12.5 μM) were mixed at room temperature in the presence of various concentrations of adenine nucleotides; the solution was immediately centrifuged at 550,000 × g for 10 min at 25 °C to sediment actin filaments. Supernatants were removed and the concentration of myosin-β was measured by ELISA.

**In Vitro Motility Assay**—We used the sliding filament assay (41) modified to use tail-specific antibodies to immobilize myosin molecules (42, 43). Coverslips coated with nitrocellulose (0.1% in isooamy alcohol) were assembled into flow chambers; chambers were sequentially incubated with 0.5 mg/ml protein A in PBS for 30 min at room temperature, block solution (1 mg/ml bovine serum albumin in PBS) for 15 min at room temperature, and 0.25 mg/ml anti-myosin-β antibody (R2652) in block solution at 4 °C overnight. This antibody recognizes the COOH-terminal 15 kDa of rat myosin-β. Subsequent steps were carried out at room temperature. Chambers were then washed with 1 mg/ml bovine serum albumin in ATPase assay solution and incubated with myosin-β (0.025 mg/ml) for 1 h. Flow chambers were washed, treated for 2 min with 20 μM rhodamine-phalloidin-labeled actin in wash buffer, and finally incubated with motility buffer (ATPase assay buffer containing 5 mM ATP, 5 mM MγC10, 10 μM calmodulin, 50 mM dithiotreitol, 0.05 mg/ml catalase, 0.25 mg/ml glucose oxidase, and 3 mg/ml glucose). Motility was observed at room temperature (23–25 °C) on an Axiovert inverted microscope, equipped with a 63 × Plan Neofluor objective and 1.6× Optivar accessory lens (all from Zeiss, Thornwood, NY). Images were captured with an intensified CCD camera (Photometas Technologies International, Monmouth Junction, NJ) using an AG-5 frame-grabber board from Scion (Frederick, MD) in an Apple Macintosh G3. Acquisition was controlled by and filament movement was measured with a modified version of NIH Image, Scion Image 1.62a (Scion). To measure actin filament velocity, we calculated the centroid position (short filaments) or the leading edge (long filaments) of each filament at 5-s intervals and averaged filament velocity over 20 frames. Data were collected from multiple protein preparations each of wild-type and Y61G myosin-β; velocities of at least 30 filaments in three separate fields were counted.

**RESULTS**

**Myosin-β Y61G Mutation**—We sought to design mutations in myosin that would render it sensitive to modified adenosine diphosphates yet maintain normal ATP hydrolysis. In a two-fold approach, we designed nucleotide analogs that were modified on the N6 amine, the furthest position on the adenine base from the 5′-triphosphate. To these nucleotide analogs, we needed to then create an additional cavity in the nucleotide-binding site of myosin. We identified amino acid positions in the binding pocket with bulky side chains that could be compacted by substitution by examining the crystal structures of *D. discoideum* myosin-II complexed with nucleotides (44–47). In each of these structures, the side chain of tyrosine-135 forms a hydrogen bond with N9 of the nucleotide (Fig. 1A). We reasoned that this tyrosine might be amenable to substitution for the following reasons. First, in some myosin isoforms, other amino acids occupy the position corresponding to Tyr-135, including leucine in *Acanthamoeba castellanii* myosin-IB, serine in *D. discoideum* myosin-IB, and phenylalanine in *Drosophila melanogaster* myosin-III (ninaC) (48). Indeed, the ATPase activity and motility of *A. castellanii* myosin-IB have been studied in detail and resemble closely those of other well characterized myosin isoforms (49, 50). Second, modifications at the C6 position of ATP are tolerated by myosin; even analogs such as 2′,3′-O-isopropylidene-6-chloropurine ribose (51) or 2-[4-azido-2-nitrophenyl]amino]ethyl triphosphate and its derivatives (52, 53) are hydrolyzed by myosin-II and support muscle contraction. These data suggest that although a hydrogen bond between Tyr-135 and a nitrogen or oxygen at the C6 position of a nucleotide may be optimal for motor activity (51), it is not essential.

We reasoned that we could substitute glycine for the equivalent residue in rat myosin-β, tyrosine-61 (Fig. 1B), and engineer a myosin mutant (Y61G) that would both hydrolyze ATP and be inhibited by N6-modified adenine diphosphates. We therefore constructed *A. californica* nuclear polyhedrosis virus recombinant baculovirus stocks capable of directing expression of wild type or Y61G rat myosin-β, each with an NH2-terminal hexahistidine tag for purification and an NH2- and COOH-terminal epitope tag for antibody detection.

**Hydrolysis of ATP by Wild Type and Y61G Myosin-β**—To produce myosin-β, we coinfected SF9 insect cells with myosin-β and *Xenopus* caldulinin baculoviruses, harvesting cells after 48 h of expression. We purified myosin-β using Ni2+-NTA chromatography and, in some cases, actin-affinity purifi-
completed in several hours. Because actin-activated ATPase activity was sufficiently low that hydrolysis of 10 μM ATP was measured within 2 days of protein purification; Y61G myosin-Iβ preparations had activities similar to those of wild type (Fig. 3; Table I). Within experimental error, concentrations of ATP and actin yielding half-maximal activity (Kₐ) of Y61G myosin-Iβ were indistinguishable.

Y61G myosin-Iβ hydrolyzed ATP with most properties very similar to those of wild type (Fig. 3, Table I). Within experimental error, concentrations of ATP and actin yielding half-maximal activity (Kₐ) of Y61G myosin-Iβ were indistinguishable. ATPase and actin-myosin binding assays generally used the Ni²⁺-NTA eluate from infected Sf9 cells (lane 1); ATP was used to release myosin from actin; purified active myosin was in the ATP supernatant (lane 9). ATPase and actin-myosin binding assays used the ATP supernatant of the actin cycling step (lane 9). A band of ~70 kDa in the ATP eluate, presumably corresponding to the contaminating band stained by Coomassie, was recognized by an antibody directed against the head of myosin-Iβ (data not shown). Although a wild-type myosin-Iβ preparation is shown here, results with Y61G myosin-Iβ were indistinguishable.

**Inhibition of ATP Hydrolysis by N⁶-substituted ATP Analogues—** To identify nucleotide analogs that bind to Y61G but not wild-type myosin-Iβ, we screened nucleotides for inhibition of [γ-³²P]ATPase activity. In a specific activity dilution experiment, unlabeled ATP inhibited [γ-³²P]ATPase activity with Kᵢ values for Y61G and wild-type myosin-Iβ of 15 μM, similar to the directly measured Kₐ values. Although ADPβS inhibited mutant and wild-type myosin-Iβ equally, ADP was a substantially less potent inhibitor of Y61G myosin-Iβ than wild type (Table II). These data are consistent with the higher ATPase activity seen with freshly isolated Y61G myosin-Iβ; more rapid ADP dissociation, consistent with the reduced affinity, could modestly accelerate ATPase activity.

The robust hydrolysis of ATP by Y61G myosin-Iβ encouraged us to screen a large collection of N⁶-modified adenine nucleotides (25, 26). By assaying with [γ-³²P]ATP, we could carry out our initial screen with the more readily available nonradioactive N⁶-modified adenosine triphosphates, even if the analogs were hydrolyzed by myosin. Using ATP near its Kₐ, we measured the inhibition of ATPase activity by a 10-fold greater concentration of a variety of analogs. Several analogs inhibited Y61G myosin-Iβ to a substantially greater degree than wild type, including those modified with a phenyl group and several analogs modified with aliphatic groups (Fig. 4, A and B).

Using a wider range of analog concentrations, we measured Kᵢ values for inhibition of ATP hydrolysis by several N⁶-modified adenosine triphosphates (Fig. 5, Table II). Our goal was not necessarily to find the most potent analogs, but rather those which are highly selective, where selectivity is defined as the ratio of Kᵢ for wild-type to Y61G Kᵢ values. ATP derivatives modified on the N⁶ position with an aromatic group were selective for Y61G over wild type, with increasing potency and selectivity as the aromatic group was moved away from the N⁶ position. Both N⁶(benzyl) ATP and N⁶(2-phenethyl) ATP were >80-fold more selective for Y61G over wild type (Fig. 5). We tried to improve selectivity by adding a substituent to various positions of N⁶(benzyl) ATP; although α-L-methyl, 2-methyl, and 4-methyl additions reduced selectivity (Fig. 4), N⁶(3-methyl benzyl) ATP was somewhat more effective than N⁶(benzyl) ATP (Fig. 5). We also found that N⁶ derivatives with aliphatic side chains were potent inhibitors of Y61G myosin-Iβ (Figs. 4 and 5).

**Table I**

Hydrolysis of ATP by wild type and Y61G myosin-Iβ

| Parameter | Wild type | Y61G |
|-----------|-----------|------|
| ATP hydrolysis rate (no actin) | 0.1 ± 0.1 s⁻¹ (n = 11) | 0.1 ± 0.1 s⁻¹ (n = 4) |
| ATP hydrolysis rate (25 μM actin) | 0.5 ± 0.3 s⁻¹ (n = 11) | 0.7 ± 0.3 s⁻¹ (n = 4) |
| Kᵢ (ATP) | 9 ± 6 μM (n = 4) | 10 ± 2 μM (n = 4) |
| Kᵢ (actin) | 10 ± 6 μM (n = 5) | 9 ± 5 μM (n = 5) |

* Assayed with 250 μM [γ-³²P]ATP. Wild-type myosin-Iβ preparations were assayed within 2 days of protein purification; Y61G myosin-Iβ activities were measured within 4 h of purification. Four wild-type myosin-Iβ preparations measured within 4 h of purification had activities of 0.1 ± 0.1 s⁻¹ without actin and 0.5 ± 0.2 s⁻¹ with actin.

* Actin concentration yielding half-maximal activity (Kᵢ).
TABLE II
Inhibition of wild type and Y61G myosin-Iβ by ATP and ADP analogs

Y61G or wild-type myosin-Iβ was assayed using 10 μM [γ-32P]ATP in the presence of 25 μM actin and 50 mM KCl. IC₅₀ values were calculated from

\[ K_i = \frac{IC_{50}}{1 + [S]/K_m} \]

where IC₅₀ is the half-blocking concentration at [S], or 10 μM [γ-32P]ATP, and Kᵢ values are taken from Table I. If not reported, n = 1. Selectivity was Kᵢ (wild type)/Kᵢ (Y61G).

| Analog              | Wild type | Y61G | Selectivity |
|---------------------|-----------|------|-------------|
|                     | μM        | μM   | fold        |
| ATP analogs         |           |      |             |
| N⁶(phenyl) ATP      | 15        | 15   | 1           |
| N⁶(benzyl) ATP      | 560       | 82   | 7           |
| N⁶(2-phenethyl) ATP | 920       | 11   | 84          |
| N⁶(3-methyl benzyl) ATP | 230  | 2.4 | 96          |
| N⁶(1-methyl butyl) ATP | 78      | 2.9 | 27          |
| N⁶(2-methyl butyl) ATP | 560     | 6.3 | 89          |
| ADP analogs         |           |      |             |
| ADP                 | 20 ± 2 (n = 3) | 74 ± 19 (n = 3) | 0.27 |
| ADP/PS              | 470       | 620  | 0.75        |
| N⁶(benzyl) ADP      | 13.3 ± 0.1 (n = 2) | 4 ± 3 (n = 5) | 43 |
| N⁶(2-phenethyl) ADP | 170 ± 40 (n = 5) | 8 ± 3 (n = 3) | 50 |
| N⁶(3-methyl benzyl) ADP | 400 ± 130 (n = 3) | 8 ± 3 (n = 3) | 50 |
| ADP                 | 195 ± 4 (n = 3) | 3.3 ± 0.4 (n = 3) | 59 |

5); the most selective of these was N⁶(2-methyl butyl) ATP. Because of poor inhibition of wild-type myosin-Iβ, selectivity values were relatively poorly constrained for some of these analogs, particularly those modified with phenyl, benzyl, 3-methyl benzyl, or 2-methyl butyl side groups.

Several of the N⁶-substituted ATP analogs were substrates for myosin-Iβ. Using a colorimetric phosphate release assay, preliminary results indicated that 100 μM N⁶(benzyl) ATP, N⁶(2-phenethyl) ATP, and N⁶(2-methyl butyl) ATP were hydrolyzed by wild-type and Y61G myosin-Iβ at velocities similar to those observed with ATP (data not shown).

Because adenosine diphosphates, rather than triphosphates, should be effective inhibitors of myosin-Iβ motor function, we synthesized several N⁶-modified adenosine diphosphates and tested their effects on ATP hydrolysis by myosin-Iβ, N⁶(benzyl) ADP, although a potent inhibitor of Y61G myosin-Iβ, affected wild-type myosin-Iβ with complex inhibitory properties and was therefore not investigated further (Fig. 6). Somewhat less selective than the triphosphates, N⁶(2-phenethyl) ADP, N⁶(3-methyl benzyl) ADP, and N⁶(2-methyl butyl) ADP nevertheless inhibited Y61G myosin-Iβ at much lower concentrations than did wild type (Fig. 6, Table II).

Actin–Myosin-Iβ Binding—Because our goal was to find an inhibitor of Y61G myosin-Iβ that tightly arrested myosin upon actin filaments, we complemented the ATPase-inhibition studies by investigating the effects of ADP analogs on the myosin-actin ATP binding equilibrium. Following incubation of actin, myosin, and nucleotides, we sedimented actin-bound myosin-Iβ by centrifugation and assayed unbound myosin-Iβ in the supernatant with an ELISA assay. Useful inhibitors should promote dissociation of Y61G myosin-Iβ with actin, even in the presence of ATP.

In the absence of ATP, wild-type myosin-Iβ bound to actin with a Kᵦ of <10 nM with or without 5 μM ADP (data not shown). ATP dissociated the actomyosin complex with Kᵦ values of ~10 μM for both wild type and Y61G (data not shown); excess ADP could reverse the dissociation elicited by ATP and drive all of the myosin into an actomyosin-ADP complex (Fig. 7A). Consistent with the reduced effectiveness of ADP for inhibition of Y61G ATPase activity, the ADP concentration required to reverse the dissociating effects of 100 μM ATP was substantially greater for Y61G than for wild-type myosin-Iβ (Fig. 7A).

The N⁶-substituted ADP analogs locked Y61G myosin-Iβ tightly to actin; N⁶(2-phenethyl) ADP, N⁶(3-methyl benzyl) ADP, and N⁶(2-methyl butyl) ADP all were significantly more potent against Y61G than wild type in reversing ATP-elicited actomyosin dissociation (Fig. 7B–D). The selectivity of N⁶(3-methyl benzyl) ADP appeared to be reduced in this assay compared with the selectivity seen in the ATPase assay; by contrast, selectivity exhibited by N⁶(2-methyl butyl) ADP and N⁶(2-phenethyl) ADP appeared to be adequate for promoting tight binding of Y61G myosin-Iβ, but not wild type, to actin filaments.

In Vitro Motility—We observed actin filament sliding on
surfaces coated with rat myosin-Ib only when we both purified myosin by actin-affinity cycling and oriented myosin molecules on the surface using a tail-specific polyclonal antibody (Fig. 8).

The actin velocity observed for wild-type rat myosin-Ib in the presence of 5 mM ATP (0.033 ± 0.007 μm s⁻¹; n = 90) was considerably slower than that reported for bovine myosin-Ib (∼0.4 μm s⁻¹; Ref. 55). Under the same conditions, actin filament velocity on Y61G myosin-Ib (0.117 ± 0.027 μm s⁻¹; n = 74) was almost 4-fold faster than on wild type. Myosin in the Ni²⁺-NTA eluate did not exhibit in vitro motility, presumably because of a large number of ATP-insensitive, actin-binding myosin molecules (see Fig. 2, lane 8).

To examine the effects of N⁶-modified analogs, we employed a lower concentration of ATP (0.5 mM) to ensure inhibition at relatively low analog concentrations. As with other myosin isozymes, even though this concentration of ATP is saturating for ATP hydrolysis (Fig. 3, Table I), motility was slowed by about 30% for wild type and 60% for Y61G myosin-Iβ as compared with the rate when 5 mM ATP was used. The apparent Kₘ values for ATP in the in vitro motility assay were 0.2 mM for wild-type and 0.8 mM for Y61G myosin-Iβ (data not shown).

When 30 or 100 μM N⁶(2-methyl butyl) ADP was included along with 0.5 mM ATP in the motility solution, actin filament velocity on surfaces coated with wild-type myosin-Iβ was unaffected; 300 μM N⁶(2-methyl butyl) ADP reduced the velocity modestly (Fig. 8, C and G). By contrast, actin velocity on surfaces coated with Y61G myosin-Iβ was completely abolished by the N⁶(2-methyl butyl) ADP, even at 30 μM (Fig. 8, F and G). Actin filaments remained unfragmented, further indicating the lack of myosin force production in the presence of the analog.

The inhibitors were effective against Y61G myosin-Iβ at higher ATP concentrations as well. Even at 2 mM ATP, 30 μM N⁶(2-methyl butyl) ADP completely arrested filament movement on Y61G myosin-Iβ (data not shown).

In the absence of N⁶(2-methyl butyl) ADP, small fractions of wild-type myosin-Iβ significantly slowed motility on mixtures of myosin-Iβ (Fig. 8H). When inhibited by N⁶(2-methyl butyl) ADP, however, Y61G myosin molecules acted in a dominant manner to block actin movement on myosin mixtures. In the presence of 2 mM ATP, actin filament movement was slowed to near zero by 100 μM N⁶(2-methyl butyl) ADP, even when Y61G myosin-Iβ was present at less than 50% of the total myosin (Fig. 8H). These data show that inhibition by N⁶-modified ADP analogs should be apparent even if the mutant myosin-Iβ molecules make up a small fraction of the total.

**Discussion**

Designing and Testing the Myosin-Iβ Y61G Mutation—To determine which roles myosin-Iβ plays in specific cellular processes, we intend to replace or supplement wild-type myosin-Iβ in cells with a mutant myosin-Iβ that we can inhibit selectively. This goal demands several features of a mutant myosin. First, in the presence of ATP alone, activities of the mutant myosin, including hydrolysis rate, unloaded velocity along actin filaments, and force production, should be as close as possible to those of wild type. Second, the mutant must be inhibited by a pharmacological agent at concentrations where the...
agent has few or no effects on the wild-type myosin. Finally, when inhibited, the mutant myosin should remain tightly bound to actin, interfering with the activity of other functionally coupled myosin molecules.

The Y61G mutation of myosin-\(\beta\) provides all three features. First, hydrolysis of ATP and chemomechanical transduction by Y61G and wild-type myosin-\(\beta\) were similar. For example, the half-maximally activating concentrations of actin and ATP concentration were nearly identical for Y61G and wild-type myosin-\(\beta\) and both translocated actin filaments. Second, several \(N^6\)-substituted adenine nucleotides were effective inhibitors of Y61G but not wild-type myosin-\(\beta\). We were able to achieve this selectivity by engineering a cavity in the nucleotide-binding site of myosin-\(\beta\), accommodating the bulky \(N^6\) substituents of the nucleotide analogs we used. The unfavorable van der Waals contacts between the \(N^6\) substituent and Tyr-61 in wild-type myosin-\(\beta\) presumably prevented these analogs from binding at high affinity. Finally, even when Y61G made up only a fraction of total myosin-\(\beta\), actin filament translocation could be fully inhibited by \(N^6\)-modified ADP analogs.

The Y61G mutation has properties that may limit its utility in vivo, however. The maximal velocity of ATP hydrolysis is higher for Y61G than wild-type myosin-\(\beta\); this feature and the diminished affinity of ADP for Y61G myosin-\(\beta\) may signal an accelerated ADP release rate. Because this rate can control the rate of myosin motility (1), more rapid ADP dissociation could account for faster actin filament translocation exhibited by Y61G myosin-\(\beta\). By decreasing the fraction of the ATPase cycle spent tightly bound (the duty ratio; see Ref. 56), accelerated ADP dissociation would also reduce the average force production of a motor. Increased unloaded velocity and decreased force production of Y61G myosin-\(\beta\) molecules might lead to unintended consequences for cellular processes requiring myosin-\(\beta\). Differences in activity between wild-type and Y61G myosin-\(\beta\) should nevertheless be useful for predicting the behavior of mixtures of myosin molecules.

Y61G Acts as a Dominant Mutation—Because myosin-\(I\beta\) molecules spend most of their ATPase cycle time detached from actin, multiple molecules must work together to generate work along actin filaments (50). Preventing dissociation from actin of a few mutant myosin-\(I\beta\) molecules should therefore halt processes dependent on ensembles of myosin-\(\beta\) molecules. Forces of \(-10\) pN are required to dissociate single, tightly bound myosin-II molecules from actin (57); because the average force

---

**Fig. 7. Effect of \(N^6\)-modified ADP analogs on ATP-actin-myosin binding.** Binding of myosin-\(I\beta\) in the N\(^6\)-NTA eluate was measured at 25 °C in the presence of 100 \(\mu\)M ATP and 12.5 \(\mu\)M actin. Results are plotted with 100% corresponding to the amount of myosin remaining in the supernatant with actin and ATP only and 0% corresponding to the amount of myosin remaining in the supernatant with actin and no nucleotides. A, for ADP, the \(K_{m,5}\) (half-maximal concentration for promoting myosin association with actin) was 85 \(\mu\)M for wild type and 940 \(\mu\)M for Y61G. B, for \(N^6(2\text{-phenethyl})\) ADP, the \(K_{m,5}\) was \(>1,000\) \(\mu\)M for wild type and 25 \(\mu\)M for Y61G. C, for \(N^6(2\text{-methyl butyl})\) ADP, the \(K_{m,5}\) was \(>2,000\) \(\mu\)M for wild type and 10 \(\mu\)M for Y61G. D, for \(N^6(2\text{-methylbenzyl})\) ADP, the \(K_{m,5}\) was \(>2,000\) \(\mu\)M for wild type and 300 \(\mu\)M for Y61G.

**Fig. 8. Inhibition of in vitro motility of Y61G myosin by \(N^6(2\text{-methyl butyl})\) ADP.** Myosin-\(I\beta\) was captured on glass coverslips with the R2652 anti-myosin-\(I\beta\) antibody; actin filaments labeled with rhodamine-phalloidin were introduced to assess myosin motility. All assays were done in the presence of 10 \(\mu\)M calmodulin, which was required for efficient motility, and 50 mM dithiothreitol, 0.05 mg/ml catalase, 0.25 mg/ml glucose oxidase, and 3 mg/ml glucose, which were required to minimize photobleaching. Because we subtracted the initial frame of a sequential collection of images from a frame 104.5 s later, the resulting panels (A–F) show movement of labeled actin filaments (63). For filaments that have not moved farther than their length (the majority), the track length is proportional to their velocity. A, surface coated with wild-type myosin-\(I\beta\), in the presence of 5 mM ATP; B, surface coated with wild-type myosin-\(I\beta\), in the presence of 0.5 mM ATP; C, surface coated with wild-type myosin-\(I\beta\), in the presence of 0.5 mM ATP and \(N^6(2\text{-methyl butyl})\) ADP. Filament movement was unaffected. D, surface coated with Y61G myosin-\(I\beta\), in the presence of 5 mM ATP. Note the longer tracks, corresponding to more rapid filament velocity. E, surface coated with Y61G myosin-\(I\beta\), in the presence of 0.5 mM ATP; \(F\), surface coated with Y61G myosin-\(I\beta\), in the presence of the 0.5 mM ATP and \(N^6(2\text{-methyl butyl})\) ADP. Filament movement was completely eliminated. Scale bar of 10 \(\mu\)M (also applies to A–E). G, filament velocity on wild-type or Y61G myosin-\(I\beta\) in the presence of 0.5 mM ATP and varying concentrations of \(N^6(2\text{-methyl butyl})\) ADP. Results plotted are mean ± S.D. of filament velocities from three to five independent myosin-\(I\beta\) preparations (for each point, \(n \geq 30\)). H, filament velocity on mixtures of wild-type and Y61G myosin-\(I\beta\). Motility carried out with 2 mM ATP; 100 \(\mu\)M \(N^6(2\text{-methyl butyl})\) ADP was also added to some samples. Results are mean ± S.D. of filament velocities from one to two independent myosin-\(I\beta\) preparations (\(n \geq 30\)); because of some day-to-day variability, all preparations were referenced to results from each preparation with 100% wild-type myosin-\(I\beta\) and no inhibitor.
production of an active myosin molecule is ~2 pN (58), a small fraction of tight myosin-actin interactions should slow or stop an ensemble.

Our data show directly that the inhibited Y61G molecules act in dominant manner to slow motility on mixtures of wild-type and mutant myosin-Iβ (Fig. 8H). Even when Y61G molecules make up <30% of the total, motility in the presence of 2 mM ATP and 100 μM N6(2-methyl butyl) ADP is slowed by ~80% compared with the control. These data show that this mutant-inhibitor pair effectually blocks myosin-Iβ-based motility and should do so even in the presence of an excess of wild-type myosin-Iβ molecules.

Y61G Myosin-Iβ in Cells—Introduction of a modest fraction of Y61G molecules into a myosin-Iβ ensemble should permit dramatic inhibition by N6-substituted ADP analogs of cellular activities dependent on the ensemble. This inhibition strategy can only be applied in certain narrowly defined circumstances, however. Because these analogs may bind to and interfere with other nucleotide-binding proteins, inhibition of these proteins must not interfere with assays for myosin-Iβ. Furthermore, delivery of the membrane-impermeable N6-substituted ADP analogs into cells may be difficult. Although microinjection could initially generate an elevated concentration of analog in a cell, enzymes such as creatine kinase, adenylate kinase, or ADP...
43. Post, P. L., Bokoch, G. M., and Mooseker, M. S. (1998) *J. Cell Sci.* **111**, 941–950
44. Smith, C. A., and Rayment, I. (1996) *Biochemistry* **35**, 5404–5417
45. Gulick, A. M., Bauer, C. B., Thoden, J. B., and Rayment, I. (1997) *Biochemistry* **36**, 11619–11628
46. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) *Biochemistry* **34**, 8960–8972
47. Bauer, C. B., Kuhlman, P. A., Bagshaw, C. R., and Rayment, I. (1997) *J. Mol. Biol.* **274**, 394–407
48. Cope, M. J. T. V., Whisstock, J., Rayment, I., and Kendrick-Jones, J. (1996) *Structure* **9**, 969–987
49. Albanesi, J. P., Fujisaki, H., Hammer, J. A., III, Korn, E. D., Jones, R., and Sheets, M. P. (1985) *J. Biol. Chem.* **260**, 8649–8652
50. Ostap, E. M., and Pollard, T. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14332–14337
51. Tonomura, Y., Imamura, K., Ikehara, M., Uno, H., and Harada, F. (1967) *J. Biochem.* **61**, 460–472
52. Wang, D., Pate, E., Cooke, R., and Yount, R. (1993) *J. Muscle Res. Cell Motil.* **14**, 484–497
53. Pate, E., Nakamaye, K. L., Franks-Skiba, K., Yount, R. G., and Cooke, R. (1991) *Biophys. J.* **59**, 598–605
54. Jontes, J. D., Milligan, R. A., Pollard, T. D., and Ostap, E. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14332–14337
55. Zhu, T., Sata, M., and Ikebe, M. (1996) *Biochemistry* **35**, 513–522
56. Howard, J. (1997) *Nature* **389**, 561–567
57. Nishizaka, T., Miyata, H., Yoshikawa, H., Ishiwata, S., and Kinosita, K., Jr. (1995) *Nature* **377**, 251–254
58. Huxley, A. F., and Simmons, R. M. (1971) *Nature* **233**, 533–538
59. Cande, W. Z. (1986) *Methods Enzymol.* **134**, 473–477
60. Gillespie, P. G., and Hudspeth, A. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2710–2714
61. Assad, J. A., and Corey, D. P. (1992) *J. Neurosci.* **12**, 3291–3309
62. Warrick, H. M., De Lozanne, A., Leinwand, L. A., and Spudich, J. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 9433–9437
63. Kinose, F., Wang, S. X., Kidambi, U. S., Moncman, C. L., and Winkelmann, D. A. (1996) *J. Cell Biol.* **134**, 885–899
64. Kapoor, T. M., and Mitchison, T. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9106–9111