Human Deafness Mutation of Myosin VI (C442Y) Accelerates the ADP Dissociation Rate*

Osamu Sato†, Howard D. White†, Akira Inoue†, Betty Belknap§, Reiko Ikebe¶, and Mitsuo Ikebe‡¶¶

From the †Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0127 and the §Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, Virginia 23507-1980

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The missense mutation of Cys442 to Tyr of myosin VI causes progressive postlingual sensorineural deafness. Here we report the effects of the C442Y mutation on the kinetics of the actomyosin ATP hydrolysis mechanism and motor function of myosin VI. The largest changes in the kinetic mechanism of ATP hydrolysis produced by the C442Y mutation are about 10-fold increases in the rate of ADP dissociation from both myosin VI and actomyosin VI. The rates of ADP dissociation from acto-C442Y myosin VI-ADP and C442Y myosin VI-ADP are 20–40 times more rapid than the steady state rates and cannot be the rate-limiting steps of the hydrolysis mechanism in the presence or absence of actin. The 2-fold increase in the actin gliding velocity of C442Y compared with wild type (WT) may be explained at least in part by the large increase in the rate of ADP dissociation. The C442Y myosin VI has a significant increase (−10-fold) in the steady state ATPase rate in the absence of actin relative to WT myosin VI. The steady state rate of actin-activated ATP hydrolysis is unchanged by the C442Y mutation at low (<10−7 M) calcium but is calcium-sensitive with a 1.6-fold increase at high (−10−4 M) calcium that does not occur with WT. The actin gliding velocity of the C442Y mutant decreases significantly at low surface density of myosin VI, suggesting that the mutation hampers the processive movement of myosin VI.

Myosin is a motor protein that interacts with actin filaments and converts energy from ATP hydrolysis into mechanical activity. Among diverse myosin superfamily proteins, class VI myosins are identified in various organisms in the animal kingdom (1–4). In mammals, myosin VI is found in various tissues, but its physiological significance is most recognized in the auditory system (5). The mouse myosin VI gene was discovered to be responsible for deafness in the Snell’s waltzer mice, which show the characteristic waltzing and circling behavior (6).

Myosin VI is expressed in both the inner and outer hair cells of the inner ear and is localized in the pericellular necklace, present between the cuticular plate and circumferential actin structures. The location suggests that myosin VI may be responsible for the maintenance of sensory hair cells. In Snell’s waltzer mice, the cochlea shows progressive loss of hair cells soon after birth (6, 7), suggesting that myosin VI function is involved in the maintenance of sensory hair cells. These findings suggest that myosin VI may function to maintain the association of the cuticular plate with the stereocilia rootlets, which in turn stabilize the hair cells (8). Although the functional deficiency of myosin VI mutation is most pronounced in auditory function, myosin VI is expressed in a variety of cell types.

The location of class VI myosin in Drosophila (Drosophila 95F myosin) in the cytoplasmic particles that move along microfilaments suggests that myosin VI is involved in the transport processes (9). In polarized cells from the intestinal brush border, myosin VI is concentrated in the terminal web with a small amount in the microvilli (10, 11) and it has been suggested that myosin VI plays a role in the endocytosis process in polarized cells. Supporting this view, the defect is most pronounced in polarized cells in myosin VI knockout mice (Snell’s waltzer mice) (12). Recently, it was found that myosin VI associates with clathrin-coated vesicles in polarized cells (11), suggesting that myosin VI plays a role in clathrin-mediated endocytosis.

The myosin VI motor domain is located at the N-terminal head domain followed by a neck domain containing one IQ motif that binds a calmodulin light chain. A potential coiled-coil domain is present at the C-terminal side of the neck region, and so it is predicted that myosin VI is a two-headed myosin. Finally, a unique globular tail domain is present at the C-terminal end of the molecule that is thought to be a targeting domain that determines the cellular binding counterpart.

Molecular characteristics of myosin VI motor function have been studied by using various techniques. The most important feature is that myosin VI moves processively (13, 14) on actin filaments toward the minus end (15), which is opposite the direction of most other myosins (4). Processive myosin motility was first found for myosin V (16, 17) and it is thought that processive myosins are more suitable for cargo movement in cells because they spend the majority of the cross-bridge cycle bound to actin (high duty ratio) and can therefore travel on actin filaments for a long distance without dissociation. Because the actin filaments in cells extend from the barbed end on the anchoring sites at the plasma membrane, these findings imply that myosin VI plays a role in cargo transport in cells from the cell surface toward the inside of the cell. The processive movement of myosin VI has been determined by direct visualization of the movement of single molecules on actin filaments with single molecule fluorescence microscopy and by mechanical assay with optical trap nanometry. This shows that successive multiple steps of single myosin VI molecules on actin filaments occur before dissociation (13, 14). The processive nature of myosin VI is also supported by the enzyme kinetic analysis of the actomyosin VI ATPase reaction (18), and it was found that ADP release from actomyosin VI was the slowest measured kinetic step of the actomyosin VI ATPase

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† To whom correspondence should be addressed: Dept. of Physiology, University of Massachusetts Medical School, 55 Lake Ave. N., Worcester, MA 01655-0127. Tel.: 508-856-1954; Fax: 508-856-4600; E-mail: Mitsuo.Ikebe@umassmed.edu.

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reaction. Myosin VI-ADP has a high affinity for actin, therefore, myosin VI spends a majority of the time bound to actin during the cross-bridge cycle.

Whereas myosin VI has been known to be responsible for deafness in mice, it has been obscure as to whether the myosin VI is linked to human hereditary hearing loss. Quite recently, it was found that the myosin VI gene is mutated in human autosomal dominant nonsyndromic hearing loss (19) and a missense mutation of C442Y in the myosin VI gene was determined to be responsible for hearing loss.

The aim of the present study is to determine the mechanoenzymatic basis of the deficiency of myosin VI function due to C442Y mutation found in human autosomal nonsyndromic hearing loss. To achieve the goal, we analyzed the critical kinetic steps of the actomyosin VI ATP hydrolysis mechanism that are likely to be important for myosin VI movement on actin filaments. We show here that the C442Y mutation increases the rate of ADP dissociation from myosin VI by ~10-fold in the presence and absence of actin and smaller changes in steady state rates of ATP hydrolysis. ADP dissociation is therefore not the rate-limiting step of the ATP hydrolysis by acto-C442Y myosin VI.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strains XL1-Blue and DH10BAC were purchased from Stratagene (San Diego, CA), and Invitrogen, respectively. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Anti-FLAG M2 affinity gel and FLAG peptide were from Sigma. Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (20). Wild type (WT)1 myosin VI HMM was basically prepared as below, and alternatively prepared as described previously (21). Smooth muscle myosin II was prepared as described (22) and phosphorylated with myosin light chain kinase (15 U/ml) in a buffer containing 50 mM KCl, 1 mM MgCl₂, 50 mM Tris-HCl (pH 7.5) at 25 °C for 15 min. mdATP, mant-ATP, and mant-ADP were synthesized from 2′-deoxy-ATP, ATP, and ADP (Sigma) and purified according to the methods of Hiratsuka (24).

Generation of the Expression Vectors for Myosin VI Constructs—Mouse cDNA clones containing nucleotides, −150 to 2565 (B10) and 1460 to 3708 (B2), in pBluescript were kindly provided by Dr. K. Avraham (Tel Aviv University). The B10 clone was digested by KpnI/PstI and the cDNA fragment of myosin VI (1-1520–1516), obtained from the B2 clone containing KpnI/PstI digestion, was inserted into the B10 clone (B2/B10). This construct, containing the entire coiled-coil domain, was used to produce recombinant baculovirus expressing myosin VI HMM containing amino acid residues Met¹–Thr²⁰⁶₂ as described previously (21, 25). A cDNA fragment containing 3156–3955 flanked with KpnI sites was produced by PCR using PstI enzyme (Invitrogen) and was ligated to a myosin VI HMM construct to produce the myosin VI full-length construct containing amino acid residues Met¹–Lys¹⁷⁰⁵. Alternatively, a new KpnI site was created to produce a cDNA fragment of myosin VI S1 containing amino acid residues Met¹–Glu¹⁶³⁰. A unique SpeI site was created at the 5′ side of the initiation codon, and then the myosin VI cDNA, cut by SpeI/KpnI digestion, was inserted into pFastBac1 (Invitrogen) baculovirus transfer vector at the polylinker region. FLAG and hexa-histidine tag sequences were introduced at the 3′ sides of the SpeI and KpnI sites, respectively. Cys⁴⁴² and/or Val⁸⁰⁰ of myosin VI constructs were mutated to Tyr and/or Trp by site-directed mutagenesis (26), respectively, and the mutation was confirmed by direct sequence analysis.

Preparation of Recombinant Myosin VI—To express recombinant myosin VI, 2 ml of S9 packed cells (about 1 × 10⁸) were co-infected with two separate viruses expressing the myosin VI heavy chain and calmodulin. The cells were cultured at 28 °C in 175-cm² flasks and harvested after 3 days. Cells were lysed by sonication in 20 ml of lysis buffer (0.1 M NaCl, 30 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 1 mM EGTA, 1 mM MgCl₂, and 1 μg/ml leupeptin). Myosin VI was eluted with buffer A containing 0.1 mg/ml FLAG peptide. 0.5–1 μg of purified protein was obtained from a 2-ml packed cell.

After SDS-PAGE analysis, fractions containing myosin VI were pooled and the protein was then concentrated with a VIVASPIN concentrator (Vivascience, Carlsbad, CA). The solvent was finally exchanged into 30 mM KCl, 20 mM MOPS-KOH (pH 7.5), 1 mM EGTA, 1 mM MgCl₂, and 5 mM β-mercaptoethanol in the concentrator. The purified myosin VI was stored in 50% glycerol and used with ATPase assay.

1 Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out on a 7.5–20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (27). Molecular mass markers used were smooth muscle myosin heavy chain (204 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myosin regulatory light chain (20 kDa), and α-lactalbumin (14.2 kDa). The amount of the myosin VI heavy chain and calmodulin was determined by densitometry as described previously (28).

ATPase Assay—The steady state ATPase activity was determined at 25 °C in the presence or absence of actin in an ATP regenerating system containing 20 units/ml pyruvate kinase and 2.5 mM phosphoenolpyruvate. The reaction was carried out in a buffer containing 30 mM KCl, 20 mM MOPS-KOH (pH 7.5), 3 mM MgCl₂, 1 mM EGTA, 2 mM ATP, 0.1 μM ATPase sites of WT or C442Y myosin VI, and actin from 0 to 64 μM. The liberated pyruvate was determined as described (29). Steady state actin-activated ATPase data were fit to steady state reaction rate constants for ATP hydrolysis and phosphate dissociation evolved, and the steady state rate constants were calculated for the first 10 s of ATP hydrolysis from differential equations describing the kinetic mechanism using least square and simplex minimization routines in the Scientist package (Micromath, Ogden, UT).

Pre-steady State Kinetic Measurements—Chemical quench measurements were done using a computer-controlled stepper motor driven quench-flow apparatus used in a pulse-quench mode as described previously (30). Stopped-flow measurements of tryptophan fluorescence enhancement were done as described previously (31). Because the myosin VI HMM does not have the conserved tryptophan residue responsible for fluorescence enhancement associated with the ATP hydrolysis step in myosin II and V, the WT and C442Y mutant were constructed with a tryptophan residue in the analogous position in the sequence (V504W). Dissociation of actomyosin VI and actomyosin VI-ADP were measured from the decrease in light scattering (32). Estimates of rate constants for ATP hydrolysis and phosphate dissociation were obtained by fitting steady state and pre-steady state kinetic data to differential equations describing the kinetic mechanism using the least square and simplex minimization routines in the Scientist package (Micromath).

In Vitro Motility Assay—The in vitro motility assay was performed as described previously in the presence of containing 25 mM EGTA, 1 mM GTP, 1 mM MgCl₂, and 5 mM β-mercaptoethanol, and 5 mM ATP (33). An appropriate concentration of WT or C442Y myosin VI full-length were attached to a coverslip, and the movement of the rhodamine-labeled actin filaments was observed. Actin filament velocity was calculated from the movement distance and the elapsed time in video images. The direction of myosin VI was determined by using rhodamine-fluorescein dual-labeled actin instead of rhodamine-labeled actin as described previously (34).

RESULTS

Expression and Purification of C442Y Myosin VI—Mouse myosin VI constructs were produced and expressed in SF9 insect cells. The constructs used in the present study are shown in Fig. 1. C442Y myosin VI S1 contains the complete head (Met¹–Glu¹⁶³⁰), C442Y myosin VI HMM contains the complete head and the entire coiled-coil domain (Met¹–Thr²⁰⁶₂), and C442Y myosin VI full-length contains the entire coding sequence (Met¹–Lys¹⁷⁰⁵). A part of the coiled-coil domain was included in the S1 construct because the inclusion of several amino acids in the coiled-coil domain stabilized calmodulin binding to myosin VI. All constructs contain N-terminal FLAG and C-terminal hexa-histidine tags to aid in purification.

The cells were co-infected with an appropriate ratio of myo-

1 The abbreviations used are: WT, wild type; mdATP, 2′-deoxy-3′-O-(N-methylanthraniloyl)-adenosine triphosphate; mdADT, 2′-deoxy-2′-O-(N-methylanthraniloyl)-adenosine diphosphate; mant-ATP, 2′,3′-O-(N-methylanthraniloyl)-adenosine diphosphate; S1, subfragment 1; HMM, heavy meromyosin; MOPS, 3-(N-morpholino)propanesulfonic acid; ATP·S, adenosine 5′-O-(thiotriphosphate).
Human Deafness Mutation of Myosin VI

Myosin VI has a single IQ motif to which a calmodulin molecule binds.

**Fig. 1. Schematic drawing of myosin VI constructs.** C442Y myosin VI HMM consists of the motor domain, neck region, and the entire coiled-coil region, whereas C442Y myosin VI S1 has a very short coiled-coil domain that is not sufficient to form a two-headed structure. C442Y myosin VI full-length contains the entire coding region containing a globular tail domain. Arrowheads indicate the position of the C442Y mutation.

sin VI heavy chain- and calmodulin-expressing viruses, and the expressed myosin VI was purified by anti-FLAG agarose affinity chromatography using the FLAG tag (see “Experimental Procedures”). Fig. 2 shows the SDS-PAGE appearance of the purified myosin VI constructs. All constructs have a high molecular mass band and a low molecular mass band and are free of 200-kDa Sf9 conventional myosin and actin. The high molecular mass bands (100, 121, and 145 kDa, for C442Y myosin VI S1, C442Y myosin VI HMM, and C442Y myosin VI full-length, respectively) were consistent with the calculated molecular mass of C442Y myosin VI S1, C442Y myosin VI HMM, and C442Y myosin VI full-length, respectively. Heavy chain bands were recognized by anti-His antibodies (Santa Cruz, Santa Cruz, CA), which indicate that each high molecular mass band is the expressed myosin VI heavy chain (not shown). The small subunits show a mobility shift with a change in [Ca^2+] that is characteristic of calmodulin (not shown).

**Dissociation of Acto-C442Y Myosin VI (ADP) by MgATP—** The $k_{obs}$ for the dissociation of acto-WT and acto-C442Y myosin VI HMM by MgATP, measured by fitting the decrease in light scattering observed upon mixing the actomyosin with MgATP, are shown in Fig. 3, A and B. The data for acto-WT and acto-C442Y myosin VI without ADP are the same within experimental error and can be extrapolated to maximum rates of $\sim 500$ s$^{-1}$ at saturating MgATP. The apparent second-order rate constants of MgATP binding to acto-WT (1.3 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$) and acto-C442Y myosin VI (1.5 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$) are $\sim 100$-fold slower than the rates measured for fast skeletal actomyosin II. Consequently, the concentrations of MgATP required to obtain the maximum rate of dissociation are much higher than normal physiological levels. Premixing acto-WT myosin VI with 0.25 mM ADP decreases the maximum rate of dissociation to 7.7 s$^{-1}$ as would be expected if dissociation of ADP from the active site limited the maximum rate of ATP binding (Fig. 3A). However, premixing acto-C442Y myosin VI with 0.25 mM ADP produces only a small decrease in the maximum rate of dissociation by MgATP (Fig. 3B, inset). The inset shows a fit of $k_{max}$ (determined at saturating [ATP]), versus ADP concentration that extrapolates to $\sim 50$ s$^{-1}$ at saturating ADP and a binding constant of ADP for actomyosin VI of 0.46 mM. The C442Y mutation affects both the rate and affinity of ADP binding to actomyosin VI, decreasing the apparent affinity by $\sim 50$-fold and increasing the dissociation rate by $\sim 7$-fold.

**ATP-induced Enhancement of Intrinsic Tryptophan Fluorescence Intensity of V504W/C442Y Myosin VI—** Myosin VI has a valine in the sequence position (Val$^{504}$) instead of the tryptophan residue, which has a fluorescence enhancement associated with the conformational change that occurs prior to or concomitant with ATP hydrolysis of other myosins (35, 36). Myosin VI shows no ATP-induced change in tryptophan fluorescence (not shown). Therefore, we mutated Val$^{504}$ of myosin VI HMM to Trp to measure the rate of the ATP-induced conformational change of myosin VI. Introduction of Trp at position 504 of myosin VI resulted in the increase in Trp fluorescence upon addition of ATP, although the observed increase (3–4%) was smaller than that for rabbit skeletal myosin S1 (20–25%). The addition of ADP did not change the intrinsic tryptophan fluorescence of the V504W mutant. MgATP produces a similar fluorescence enhancement in V504W/WT (not shown) and V504W/C442Y myosin VI HMM (Fig. 4A). The dependence of $k_{obs}$ upon ATP concentration is shown in Fig. 4B. The apparent second-order rate constants of MgATP binding are 6 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ for WT (not shown) and 5 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ for C442Y myosin VI. The maximum rates at saturating ATP are the same within experimental error for V504W/WT (53 s$^{-1}$) and V504W/C442Y myosin VI (50 s$^{-1}$) and can either be interpreted as a measure of the hydrolytic step or of a conformational step preceding hydrolysis. We cannot accurately identify...
the data are fits to the equation:

$$k_{obst} = k_{max}/(1 + [MgADP])$$

where $k_{max}$ is the maximum observed rate constant, $[MgADP]$ is the concentration of MgADP, and $k_{obst}$ is the observed rate constant. The $k_{max}$ values were obtained by fitting the time dependence of the decrease in light scattering to $I(t) = I_0 e^{-k_{obst}t + C}$. Solid lines through the data are fits to the equation: $k_{obst} = k_{max}/[MgATP]$. A, fit values for acto-WT myosin VI HMM in the absence of ADP (open circles): $k_{max} = 500 \text{s}^{-1}$ and $k_{app} = 38.4 \text{mM}$, and in the presence of 0.25 mM MgADP (open triangles): $k_{max} = 7.7 \text{s}^{-1}$ and $k_{app} = 63.3 \text{mM}$. B, fit values for acto-C442Y myosin VI HMM in the absence of ADP (solid circles): $k_{max} = 500 \text{s}^{-1}$ and $k_{app} = 34.3 \text{mM}$, and in the presence of 5.0 mM MgADP (solid triangles): $k_{max} = 92 \text{s}^{-1}$ and $k_{app} = 24.3 \text{mM}$. Inset, the $k_{max}$ values from the experiments in which the acto-C442Y myosin VI initially contained 0, 0.25, 1.0, and 5.0 mM MgADP were fit by a hyperbolic equation: $k_{max} = (k_{TA} + k_{AD})/(k_{AD} + [ADP])$, where $k_{TA}$ = 0.46 mm, $k_{AD}$ = 4.9 mm, and $k_{AD} = 500 \text{s}^{-1}$. Experimental conditions: 30 mM KCl, 20 mM MOPS, 1 mM MgCl$_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol (pH 7.5), 25°C.

The results of V504W/WT and V504W/C442Y myosin VI demonstrate that the C442Y mutation does not significantly alter the rates of the initial steps of the ATP hydrolysis mechanism.

Kinetics of Product Dissociation from (Acto) C442Y Myosin VI—It has been reported that the rate of ADP dissociation from actomyosin VI is slow and it was proposed that ADP dissociation is the rate-limiting step of the actomyosin VI ATPase cycle (18). Here we report measurements of the rates of dissociation of mant-ADP from (acto) C442Y myosin VI HMM to be at least 9–10-fold more rapid than from (acto) WT myosin VI HMM. Fig. 5A shows the dependence of $k_{obs}$ of mant-ADP binding to WT (open symbols) and C442Y myosin VI (solid symbols) upon [mant-ADP]. The values obtained by extrapolation of the binding rate to zero mant-ADP concentration are in good agreement with the dissociation rate obtained by mixing the myosin VI-mant-ADP complex with excess ATP. The dissociation rate of mant-ADP from C442Y myosin VI, 55 s$^{-1}$, is almost 10-fold faster than WT, 5.7 s$^{-1}$ (Fig. 5D). The rate constant for mant-ADP binding is also significantly increased in C442Y myosin VI (Fig. 5A). These results indicate that the C442Y mutation does not significantly change the affinity for mant-ADP but significantly increases the association and dissociation rates for mant-ADP. The rates of mant-ADP dissociation from acto-WT and acto-C442Y myosin VI were measured from the rates of dissociation of the ternary actomyosin VI-mant-ADP complexes by MgATP to be 6.8 s$^{-1}$ for acto-WT and 63 s$^{-1}$ for acto-C442Y myosin VI (Fig. 5D). The rates of dissociation of mant-ADP
from C442Y myosin VI are not significantly increased by actin as was previously observed for T406A(E) myosin VI S1 (18).

We have also measured the kinetics of product release by double mixing experiments in which the myosin is mixed with substrate, incubated in a delay line for a few seconds to allow for substrate binding and hydrolysis to occur after which the myosin nucleotide complex is mixed with actin to determine the rate of product dissociation. Such experiments have been used to measured the rate of phosphate dissociation from actomyosin-ADP-Pi, for myosins II, V, and VI using fluorescent phosphate-binding protein (18, 32, 37–40). Although we were able to measure the rate of phosphate dissociation for acto-WT myosin VI-HMM-ADP-Pi, experiments to measure the rate of phosphate dissociation from acto-C442Y myosin VI HMM were “unsuccessful,” presumably because the slow rate of ATP binding and rapid steady state did not produce a sufficiently high concentration of C442Y myosin VI-ADP-Pi in the first mixture, and only a slow signal limited by the rate of ATP binding to actomyosin was observed. This result is consistent with the results from the quench-flow experiments in Fig. 6, which indicate that only a few percent of the bound nucleotide would be present as the C442Y myosin VI-ADP-Pi, intermediate as compared with >60% for myosins V and II and ~20% for WT myosin VI.

We were, however, able to observe product dissociation by initially mixing C442Y myosin VI HMM with a 5-fold molar excess of mdATP. The more rapid rate of mdATP binding and substrate concentrations higher than the myosin site concentration produce a significantly higher steady state concentration of C442Y myosin VI-HMM-mdADP-Pi. Excess (2 mM) MgATP (non-fluorescent) was included with the actin to prevent the excess mdATP from binding to actomyosin after the second mixture.

The rate of product dissociation from acto-C442Y myosin VI-HMM-mdADP-Pi, $30 \text{ s}^{-1}$ (data not shown), is similar to the rate of phosphate dissociation observed from acto-WT myosin VI-HMM-ADP-Pi, using phosphate-binding protein (18) and is slightly slower than that observed directly for mant-ADP dissociation from acto-C442Y myosin VI HMM (Fig. 5D). This indicates that the rate of phosphate dissociation from acto-C442Y myosin VI-ADP-Pi is at least as fast as from acto-WT myosin VI-ADP-Pi. A similar experiment used to measure product dissociation from acto-WT myosin VI-mdADP-Pi, measured at a rate of $\sim 5 \text{ s}^{-1}$, which is consistent with the slower rate of ADP dissociation from acto-WT myosin VI.

Quench-flow Measurements of ATP Binding to and Hydrolysis by C442Y Myosin VI—The slow rate of ATP binding to myosin VI makes measurement of a phosphate burst especially difficult. A phosphate burst can be observed using submicro-molar concentrations of myosins II and V where the second-order rate constant of ATP binding is $>10^6 \text{ M}^{-1} \text{s}^{-1}$ and the steady state rate is $<0.1 \text{ s}^{-1}$. The second-order rate constant of ATP binding to myosin VI, $\sim 5 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, is $\sim 25$ times slower than a myosin site concentration of $25 \mu \text{M}$ required to make the determination. Although we were unable to obtain such a high concentration of myosin VI HMM sites, we were able to obtain the required concentration of the smaller myosin VI S1 construct, equivalent to subfragment 1 of other myosins. At the highest concentrations that we were able to obtain with myosin VI S1 (24 $\mu \text{M}$ S1 after mixing with ATP) the rate of binding substoichiometric ATP (calculated from the

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*H. White, unpublished data.*
of ATP. The relatively low affinity of ADP for myosin VI, were bound to the active site, it would prevent the rapid binding phophate burst. If a slowly dissociating molecule (most likely ADP)

Equilibrium constants predict 0.13 of the ATP to be hydrolyzed in the phosphate burst of 20% of ATP. The same assumptions for rate and the tryptophan fluorescence enhancement (Fig. 4B) observed for WT myosin VI, −50 s⁻¹, is much faster than the rate of ADP dissociation, −6 s⁻¹. A second possibility is that only a small fraction (less than 20%) of the protein is catalytically active. We consider this to be unlikely for two reasons: 1) the steady state rates are very reproducible between preparations as shown in Fig. 7 and 2) single and multiple turnover hydrolysis of mdATP by myosin VI are consistent with an active site concentration equal to the protein concentration (data not shown).

Therefore, the absence of a phosphate burst is best explained by a mechanism in which either the equilibrium constant of the hydrolytic step is significantly less than 1 or the hydrolytic step is rate-limiting. Relatively low $K_{cat}$ values have been reported for several other types of myosin (18, 38, 40–42). A second approach to analyzing the quench-flow data is to fit the data to a kinetic mechanism (Equation 1) in which the rate constants $k_T$ and $k_D$ are fixed to the values determined in independent experiments (Figs. 4 and 5) and $k_H$ and $k_{DP}$ were varied using the simplex algorithm to obtain the best fit of the data. Solid lines through the data are the best global fits of the rate constants to the data at three concentrations of protein and ATP for WT (Fig. 6A) and C442Y myosin VI (Fig. 6B).


Human Deafness Mutation of Myosin VI

ATPase assays of myosin VI constructs were performed in the presence of 1 mM EGTA or 0.1 mM CaCl₂ as described in the legend to Fig. 7. The parameters were calculated from the data based on the equation, $V = V_{\text{max}} \cdot [\text{actin}] / (k_{\text{actin}} + [\text{actin}]) + V_D$.

### Table I

|                      | $K_{\text{actin}}$ (μM) | $V_0 + V_{\text{max}}$ (s⁻¹ head⁻¹) |
|----------------------|-------------------------|--------------------------------------|
| 1 mM EGTA            |                         |                                      |
| WT myosin VI S1      | $7.4 \pm 0.7$ (3)       | $2.95 \pm 0.06$ (3)                  |
| C442Y myosin VI S1   | $2.3 \pm 0.5$ (3)       | $3.12 \pm 0.08$ (3)                  |

|                      | $K_{\text{actin}}$ (μM) | $V_0 + V_{\text{max}}$ (s⁻¹ head⁻¹) |
|----------------------|-------------------------|--------------------------------------|
| 0.1 mM CaCl₂         |                         |                                      |
| WT myosin VI S1      | $8.7 \pm 1.0$ (3)       | $3.57 \pm 0.09$ (3)                  |
| C442Y myosin VI S1   | $4.4 \pm 0.4$ (3)       | $5.05 \pm 0.08$ (3)                  |

* Shown are mean ± S.E. and number of data in parentheses.

### Table II

|                      | $K_{\text{actin}}$ (μM) | $V_0 + V_{\text{max}}$ (s⁻¹ head⁻¹) |
|----------------------|-------------------------|--------------------------------------|
| 1 mM EGTA            |                         |                                      |
| WT myosin VI S1      | $0.18 \pm 0.02$ (5)     | $0.082 \pm 0.004$ (5)                |
| C442Y myosin VI S1   | $1.77 \pm 0.09$ (5)     | $1.29 \pm 0.14$ (5)                  |

* Shown are mean ± S.E. and number of data in parentheses.

$k_{-H}$ was determined from the measurement of $k_{H} + k_{-H} = 50$ s⁻¹ from the rate of the tryptophan fluorescence enhancement at saturating MgATP as in Fig. 4. The rate constants corresponding to the solid lines through the data are summarized in the columns of Table III labeled “WT-QF and C442Y-QF.” The best fit for the equilibrium constant of the ATP hydrolysis step is 0.2–0.5. A comparison of the mechanisms of WT and C442Y myosin VI indicate that the increased steady state rate in the absence of actin is primarily due to a 5-fold increase in the rate of phosphate dissociation from C442Y myosin VI-ADP-P, to $\sim 2$ s⁻¹. The observed steady state rate is the product of the unfavorable equilibrium constant of ATP hydrolysis while bound to myosin, $K_H$, and the phosphate dissociation rate, $k_{-\text{DAP}}$.

Steady State Kinetics of the ATPase Activity of C442Y Myosin VI—Steady state ATP hydrolysis measured with WT and C442Y myosin VI S1 using a linked assay to avoid possible inhibition by ADP produced by ATP hydrolysis. The basal ATPase activity of C442Y myosin VI was much higher than that of WT both at low and high [Ca²⁺] (Fig. 7, Tables I and II). The ATPase activity of C442Y myosin VI S1 in the presence of 1 mM EGTA was increased at saturating actin by a maximum rate of −1.4 s⁻¹ above basal rate. Ca²⁺ (0.1 mM) increases the $V_{\text{max}}$ of the actin-activated ATPase of C442Y myosin VI, −3.8 s⁻¹ above basal rate. It should be noted that the Ca²⁺ induced increase in the ATPase activity of the C442Y mutant is not because of the dissociation of bound calmodulin based on the result of the actin cosedimentation assay (data not shown). Similar $V_{\text{max}}$ and $K_{\text{actin}}$ values were obtained for C442Y myosin VI S1 and HMM (not shown).

Steady state ATPase data obtained with (C442Y) myosin VI S1 were a fit to the ATP hydrolysis mechanism shown in Table III using the Scientist package to obtain the best fit of the rate constants to the steady state data (shown in bold in the table). Other rate constants (shown in italics), which were held constant during the fitting procedure, were determined as described in the footnote of Table III. The values obtained for $k_{H}$ and $k_{-\text{DAP}}$ are consistent within a factor of two of the determinations from quench-flow data, indicating good internal consistency of the data and fitting procedures. The equilibrium constant for the hydrolysis step in the absence of actin, $K_H$, can be determined from $k_{H}/k_{-H}$, where ($k_{-H} = 50 − k_{H}$) to be 0.35–0.5 and is not significantly affected by the C442Y mutation as shown in the legend of Table III. The rate constant for phosphate dissociation in the absence of actin is increased −20-fold in C442Y myosin VI, which is similar to the −10-fold increase in the rates of ADP dissociation from C442Y myosin VI. The rate of attached hydrolysis, $k_{\text{ATP}}$−5 s⁻¹ is, the rate-limiting step at saturating actin for C442Y myosin VI and is partially rate-limiting for WT myosin VI. Although the apparent affinity of M-ATP and M-ADP-P for actin, $K_{\text{TAD}} = 500 k_{\text{TAD}}$, decreases from 25 μM for WT to 10 μM for C442Y myosin VI, the apparent second-order rate constant for phosphate dissociation, $K_{\text{TAD}} k_{-\text{DAP}}$, is the kinetically important parameter. However, the evidence that $k_{-\text{DAP}}$ is unchanged in C442Y myosin VI is indirect and the data could be equally well explained by a 2.5-fold increase in $k_{-\text{DAP}}$ rather than $K_{\text{TAD}}$. The important information derived from analysis of the steady state data is: 1) confirmation of the small equilibrium constant of the hydrolysis step, $K_H < 1$, in the absence of actin, which is also indicated by the quench-flow data; 2) the bound hydrolysis step is rate-limiting at high actin for C442Y myosin VI and partially rate-limiting for WT myosin VI, and 3) the C442Y mutation increases the rate of the phosphate dissociation step, $k_{-\text{DAP}}$, 5-20-fold in the absence of actin.

Actin Gliding Activity of C442Y Myosin VI—Fig. 8 shows the actin gliding velocity on C442Y myosin VI full-length-coated coverslips. The maximum velocity of C442Y myosin VI, 0.6 μm/s, is twice that measured for the WT myosin VI, 0.3 μm/s. The larger value could be explained in part by the higher rate of ADP dissociation from acto-C442Y myosin VI, which is 10-fold larger, but it would appear that ADP dissociation must not be the only kinetic step that affects the rate of C442Y myosin VI motility. The dependence of the motility at submaximal MgATP concentration ($k_{\text{actin}} / K_{\text{app}}$) can be used to estimate the average distance, $d$, over which the cross-bridge remains attached to actin during the power stroke according to Equation 2 (31) to be −14 nm.

$$d = (k_{\text{actin}} / K_{\text{app}} / k_{\text{H}}) = (0.6 \mu\text{m s}^{-1} \cdot 2.8 \times 10^{-3} \text{m}) / 1.5 \times 10^{-9} \text{m}^2 \text{s}^{-1} = 14 \text{ nm} \quad (\text{Eq. 2})$$

This value can be compared with the value determined for WT myosin VI of −45 nm by using the same calculation and by single molecule methods to be 36 nm (14). A marked increase in the ADP off-rate implies that the duty ratio of C442Y myosin VI is lower than that of the WT. Fig. 9 shows the actin gliding velocity as a function of the surface density of myosin VI. Whereas the actin gliding velocity remained constant at low surface density for WT myosin VI full-length, the velocity was significantly decreased at low surface density for the C442Y mutant. The requirement of a higher myosin density to obtain maximal velocity suggests that the C442Y mutation hampers the processive nature of myosin VI movement.

Fig. 10 shows the movement of dual labeled actin filaments on a C442Y myosin VI-coated coverslip. This result indicates that C442Y myosin VI moves actin filaments with the barbed end at the front of the movement and does not alter the direction of myosin VI motility.
DISCUSSION

Myosin VI is involved in a hearing disorder in mice and a null mutation of myosin VI, known as Snell’s waltzer, which causes congenital deafness and exhibits a characteristic circulating behavior (5). Recently, human autosomal dominant hearing loss has been found to be because of a missense mutation of human myosin VI at Cys442 (19). This work has been done to characterize the affects of the C442Y mutation upon the ATP hydrolysis mechanism.

Previously, it was proposed that ADP dissociation is the rate-limiting step for the wild type actomyosin VI ATPase reaction because the rate constant of the ADP dissociation step was similar to the \(V_{\text{max}}\) of actomyosin VI steady state ATP hydrolysis. It was therefore proposed that the slow ADP dissociation step is critical for the processive motor properties of myosin VI (18). Recent studies have indicated that myosin VI is a processive motor that moves on actin filaments without dissociation for a long distance with a large step size that could reach across the helical pitch of the actin filament (13, 14).

We found three major changes in mechanoenzymatic func-

| Myosin VI Method | WT QF | WT SS | C442Y | C442Y SS |
|------------------|------|------|-------|---------|
| \(k_{\text{AT}}\)  | \(\mu M^{-1} s^{-1}\) | 0.013 | 0.015 |
| \(k_{\text{AH}}\)  | \(s^{-1}\) | 6 ± 2 | 5 ± 1 |
| \(k_{\text{AD}}\)  | \(s^{-1}\) | 12 ± 4 | 14 ± 2 |
| \(k_{\text{DAP}}\) | \(s^{-1}\) | 30  | >30 |
| \(k_{\text{DPA}}\) | \(s^{-1}\) | 8  | 50 |
| \(k_{\text{H}}\)   | \(\mu M^{-1} s^{-1}\) | nd | 0.1 |
| \(k_{\text{H(Dynamic)}}\) | \(s^{-1}\) | 6.8 | 63 |
| \(k_{\text{H(Native)}}\) | \(\mu M^{-1} s^{-1}\) | 0.25  | 1.9 |
| \(k_{\text{T}}\)   | \(s^{-1}\) | 10 ± 2 | 17 ± 2 |
| \(k_{\text{T}}\)   | \(s^{-1}\) | 16 ± 7 | 13 ± 1 |
| \(k_{\text{H}}\)   | \(s^{-1}\) | 53  | 50 |
| \(k_{\text{H}}\)   | \(s^{-1}\) | 0.24 | 0.51 |
| \(k_{\text{DPA}}\) | \(s^{-1}\) | 0.47 | 0.36 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 51 ± 14 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 20 ± 4 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 0.40 ± 0.1 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 0.43 ± 0.1 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 2.2 ± 2 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 8 ± 1 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 5.7  | 5.5 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 0.34 | 3.9 |
| \(k_{\text{DPA}}\) | \(\mu M^{-1} s^{-1}\) | 0.06 | 0.04 |
WT and C442Y myosin VI. Surface density was determined by denaturation system (20 units/ml pyruvate kinase and 2.5 mM phosphoenolpyruvate). The bars represent the standard deviation with 10 actin filaments observed for motility assay. Solid lines were obtained according to the equation: 

\[ V = \frac{V_{\text{max}}(\text{ATP})}{K_{\text{ATP}} + [\text{ATP}]} \]

where \( k_{\text{ATP}} \) and \( V_{\text{max}} \) of 0.51 mM and 0.31 \( \mu \)m/s for WT, and 2.8 mM and 0.64 \( \mu \)m/s for C442Y myosin VI full-length, respectively.

First, we found that the ADP dissociation rate is markedly increased by the C442Y mutation. Cys442 lies at the end of the \( \alpha \)-helix that connects the myopathy loop and switch II loop by analogy to the structure of myosin II motor domain (43). Therefore, it is not surprising that C442Y changes the release of bound ADP because of its location near the switch II loop that is known to alter ADP dissociation rates from actomyosin.

The rate constant of ADP dissociation from acto-C442Y myosin VI, \( >50 \text{ s}^{-1} \), is much faster than the steady state ATP hydrolysis rate, \( \sim 3 \text{ s}^{-1} \). ADP dissociation clearly is not the rate-limiting step for ATP hydrolysis by C442Y myosin VI nor is phosphate dissociation likely to be as both dissociate at rates greater than or equal to 30 \( \text{s}^{-1} \) from acto-C442Y myosin VI-ADP-P\(_i\). Quench-flow data and global fitting of the actin-activated ATP hydrolysis data indicate that attached ATP hydrolysis (AM-ATP \( \rightarrow \) AM-ADP-P\(_i\)) is rate-limiting for C442Y myosin VI and partially rate-limiting for WT myosin VI at saturating actin. It therefore might be expected that C442Y myosin VI should be a less processive motor than WT myosin VI because the ADP dissociation rate is more than 10 times faster than the steady state hydrolysis rate.

Second, the C442Y mutation markedly increased the basal ATPase activity of myosin VI. Whereas the rate constant of the ADP dissociation step is significantly increased in C442Y myosin VI, this is unlikely to be responsible for the increase in basal ATPase activity by C442Y mutation because the rate constants of ADP dissociation are much faster than the basal ATPase activity of both WT and C442Y mutant. However, the rate constant of phosphate dissociation is accelerated -10-fold by the C442Y mutation and it is likely that this is a result of the same alternation in the myosin VI structure as the increase in ADP dissociation. However, neither the ATP binding rate nor the rate of ATP-induced change in conformation probed by the intrinsic tryptophan fluorescence change is significantly affected by the C442Y mutation. These steps are also much faster than the overall hydrolysis rate at physiological ATP concentration. We have used a minimal mechanism in Equation 1 and Table III to fit the kinetic data, which assumes that the tryptophan fluorescence change is a measure of the rate of the hydrolysis step. The data fit equally well by a mechanism in which a rapid conformational step \( k_{\text{T}} + k_{-\text{T}} = 50 \text{ s}^{-1} \) precedes a slow hydrolytic step as shown in Equation 3.
Both kinetic mechanisms are significantly different from striated myosins in which rapid and irreversible ATP binding is followed by rapid hydrolysis with an equilibrium constant in favor of the M-ADP-P. The kinetic data for C442Y and WT myosin VI indicate that ATP binding to myosin VI is much slower than for most other myosins. The hydrolysis step is either unfavorable and/or the rate-limiting step in the presence and absence of actin.

Actin increases the steady state rate of myosin VI ATP hydrolysis primarily by increasing the rate of the phosphate dissociation step (Table III), whereas the actomyosin-ADP dissociation rate is very little by actin for both WT and C442Y myosin VI. The maximum rates of acto-activated ATP hydrolysis are not significantly increased for C442Y myosin VI even though the ADP dissociation rate is increased -10-fold. This indicates that ADP dissociation is clearly not rate limiting for acto-C442Y myosin VI and is only partially rate limiting for acto-WT myosin VI. The results imply that the C442Y mutation significantly decreases the duty ratio of myosin VI. In addition, the rapid dissociation of products from C442Y myosin VI in the absence of actin will reduce the concentration of the myosin-ADP-P intermediate available to produce a power stroke. Consistent with this notion, we found that the actin sliding velocity by C442Y myosin VI full-length decreased at low surface density of myosin VI in contrast to the wild type myosin VI.

Third, the acto-activated ATPase activity of C442Y myosin VI is Ca2+ sensitive. Cys442 is located in the motor core domain and quite distal from the calmodulin light chain binding site on the neck, therefore, it is unlikely that C442 directly interacts with the calmodulin molecule. Regulation of the ATPase activity by the distal regulatory site is also known for smooth muscle and non-muscle myosin II in which the acto-activated ATPase activity is regulated by phosphorylation of the regulatory light chain associated at the C-terminal end of S1 (44–47). In the case of myosin II, phosphorylation-mediated regulation requires a two-headed structure and it is thought that inter-head interaction is involved in the regulation mechanism (48–53). Here, we found that a single headed C442Y myosin VI is also sensitive to Ca2+ although the magnitude of regulation was much smaller than that of phosphorylation of myosin II. This indicates that inter-head interaction is not critical for the Ca2+-dependent regulation of C442Y myosin VI. Ca2+ -induced activation of the acto-activated ATPase activity has been reported for other unconventional myosins (28, 53–58). Because bound calmodulin can be dissociated from the heavy chain at high Ca2+, it was proposed originally that the activation of the ATPase activity is because of the dissociation of calmodulin (53, 56). Subsequently, it was shown that the change in the ATPase activity occurs at relatively low Ca2+ (pCa6) where there was no detectable decrease in bound calmodulin, suggesting that the Ca2+ effect is not directly related to the physical dissociation of calmodulin (28, 59). The present result is consistent with a mechanism in which C442Y myosin VI shows increased ATPase activity at high Ca2+ without a change in bound calmodulin.

Human DFNA22 is a dominant mutation (19), whereas the Snell’s waltzer myosin VI null mutation is recessive. This suggests that the C442Y missense mutation interferes with the function of the wild type myosin VI in cells, resulting in a dominant mutation. It would be anticipated that the heterozy-
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Human Deafness Mutation of Myosin VI (C442Y) Accelerates the ADP Dissociation Rate
Osamu Sato, Howard D. White, Akira Inoue, Betty Belknap, Reiko Ikebe and Mitsuo Ikebe

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