Lethal Kinesin Mutations Reveal Amino Acids Important for ATPase Activation and Structural Coupling

Katherine M. Brendza§‡, Debra J. Rose‡, Susan P. Gilbert¶, and William M. Saxton‡**

From the §Department of Biology, Jordan Hall, Indiana University, Bloomington, Indiana 47405 and the ¶Department of Biological Sciences, Langley Hall, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

To study the relationship between conventional kinesin's structure and function, we identified 13 lethal mutations in the Drosophila kinesin heavy chain motor domain and tested a subset for effects on mechanochemistry. S246F is a moderate mutation that occurs in loop 11 between the ATP- and microtubule-binding sites. While ATP and microtubule binding appear normal, there is a 3-fold decrease in the rate of ATP turnover. This is consistent with the hypothesis that loop 11 provides a structural link that is important for the activation of ATP turnover by microtubule binding. T291M is a severe mutation that occurs in α-helix 5 near the center of the microtubule-binding surface. It impairs the microtubule-kinesin interaction and directly effects the ATP-binding pocket, allowing an increase in ATP turnover in the absence of microtubules. The T291M mutation may mimic the structure of a microtubule-bound, partially activated state. E164K is a moderate mutation that occurs at the β-sheet 5α/loop 8b junction, remote from the ATP pocket. Surprisingly, it causes both tighter ATP-binding and a 2-fold decrease in ATP turnover. We propose that E164 forms an ionic bridge with α-helix 5 and speculate that it helps coordinate the alternating site catalysis of dimerized kinesin heavy chain motor domains.

Conventional kinesin is an abundant microtubule motor protein that functions in a number of important intracellular transport processes (reviewed in Ref. 1). It is a heterotetramer comprised of 2 kinesin heavy chains (KHCs) and 2 light chains. The heavy chains dimerize to form an elongated stalk with 2 amino-terminal globular domains ("motor domains") at one end and the light chains at the other end. The light chains and stalk are expected to bind the cytoplasmic cargoes that conventional kinesin transports. Each motor domain couples a cycle of ATP turnover to conformational changes and a cycle of microtubule binding and release that generates displacement toward the microtubule plus end. A single motor domain is not processive (2, 3). In contrast, dimerized motor domains are remarkably processive, making hundreds of steps before releasing from the microtubule (4, 5). This feature is probably critical for the resolute transport of small organelles that can present only one or a few kinesin molecules to a microtubule.

The pathway of ATP hydrolysis has been studied in detail, and there is general agreement on a number of features of kinesin's alternating site mechanism (6–13). The model in Fig. 1 illustrates some of those features in the context of its mechanochemical cycle. Free in solution, kinesin exists with ADP bound in its active site. The ATPase cycle begins as one motor domain binds to the microtubule, leading to rapid release of ADP (step 1). ATP binding at this empty active site (step 2) stimulates binding of the second motor domain to the microtubule and release of its ADP (step 3). Thus, the two motor domains of the dimer are coupled through this step. After ATP hydrolysis by the first head (step 4), a rate-limiting step occurs (step 5) encompassing both detachment from the microtubule and phosphate release. ATP binding to the second head and rebinding of the first head to the next forward site on the microtubule lattice 16 nm away advances the kinesin dimer center of mass by 8 nm (step 2; Ref. 14). For KHC, recent evidence has ruled out step distances other than 8 nm and coupling ratios other than 1 ATP per 8 nm step (15–17).

The structure of the KHC mechanochemical domain in both monomer and dimer forms has been studied by x-ray crystallography and electron microscopy (18–29). The crystallographic analysis of KHC monomers with ADP bound revealed an extended β-sheet flanked on each side by 3 α-helices (see Fig. 4B). The nucleotide-binding site is near the top of the sheet on the side occupied by helices 1–3. Evidence to date indicates that the binding of kinesin to microtubules is mediated by multiple contacts on the side of the molecule occupied by helices 4–6. These studies include structural evidence based on electron micrographs of microtubule-KHC complexes (18–20, 22, 23, 25, 28, 30). Crystallographic studies of rat KHC dimers with ADP bound showed the same motor domain structure seen with monomers and revealed that the heads are joined by coiled-coil interactions at α7. The dimer structure also revealed that a negatively charged portion of L8b on one head can approach a positively charged portion of L10 on the other head (Fig. 8). The opposing charges and proximity of these loops has raised the possibility of a salt bridge forming between the motor domains that may be important for the coordination of their mechanochemical cycles and consequently for the processive movement of kinesin.

To gain insight into how kinesin moves, the effects of specific
The cycle repeats for the second head, resulting in 2 steps driven by the hydrolysis of 2 ATPs. No nucleotide at active site. Available microtubule-binding site is rapid (step 2 and 3) and advances the center of mass of the dimer by 8 nm, thereby completing one step cycle. Nucleotide state of each is different. Step 5 is the slowest in the pathway, encompassing both detachment of the motor domain from the microtubule (step 3). Subsequently, ATP hydrolysis (step 4) occurs forming an intermediate in which both motor domains are bound to the microtubule but the hinge I is important for normal motor domain mechanochemistry (32). Changes in hinge I, which connects the neck to the beginning of the kinesin stalk, can alter the velocity and processivity. Changes in hinge I, other neck sequences can alter processivity but do not abolish it, suggesting that structural changes on the mechanochemistry of KHC have been studied. Woehlke et al. (31) have discovered a number of probable microtubule-KHC contact sites by changing solvent-exposed, polar amino acids to alanine and testing for effects on both motility and microtubule stimulated ATPase activity. Several of those sites are concentrated in L11, α4, L12, and α5. Two additional in vitro mutagenesis studies have focused on the contributions of the KHC hinge I and neck regions to motor velocity and processivity. Changes in hinge I, which connects the neck to the beginning of the kinesin stalk, can alter the velocity of gliding movements dramatically, indicating that hinge I is important for normal motor domain mechanochemistry (32). Changes in α7 and other neck sequences can alter processivity but do not abolish it, suggesting that structural interactions between dimerized motor domains outside the neck region also contribute to processivity (33).

We have addressed questions of KHC structure-function using an approach that combines in vivo genetics and in vitro mechanochanical tests. We reasoned that by screening for missense mutations that disrupt axonal transport (34) and cause lethality in Drosophila, amino acid substitutions would be identified that affect kinesin mechanochemistry in ways that are physiologically relevant. By testing purified mutant motor domain dimers for motility, microtubule interaction, nucleotide-binding, and steady-state ATP turnover, we have identified two amino acids that are important for communication from the microtubule-binding site to the nucleotide-binding site and one likely to form an ionic bridge between β5α/γ5δβ and α5; a bridge that influences nucleotide binding and may be involved in coordinating the ATPase and microtubule-binding cycles of dimerized heads.

**Experimental Procedures**

**Materials—**[α-32P]ATP (>3000 Ci/mmol) was from NEN Life Sciences (Boston, MA); polyethyleneimine (PEI)-cellulose TLC plates (EM Science of Merck, 20 × 20 cm, plastic backed) were from VWR Scientific (Bridgeport, NJ); Taxol (Taxus brevifolia) was from CalBiochem-Nove Biochem International (San Diego, CA); QiAmp Tissue Kit and Qiaquick gel extraction kit were from Qiagen (Valencia, CA); Thermo Sequenase sequencing kit was from Amersham Pharmacia Biotech, T4 DNA ligase and restriction enzymes were from New England Biolabs (Beverley, MA), Chameleon Double-Stranded Site-Directed Mutagenesis Kit was from Stratagene, Inc. (La Jolla, CA), ATG, GTP, AMP-PNP, S-Sepharose and DEAE-Sepharose were from Amersham Pharmacia Biotech, Bio-Rad Protein Assay, ovalbumin, and IgG were from Bio-Rad.

**Genetics—**Drosophila were cultured at 25 °C with a 12-h light and 12-h dark cycle on standard soft medium (0.5% agar, 7% molasses, 6% cornmeal, and 0.8% killed yeast) seeded with live yeast. Descriptions of some of the mutations used in this study can be found in Lindaley and Zimm (35). Description of Khc-Khc; can be found in Saxton et al. (36). All other Khc alleles were isolated in a standard F2 screen for recessive lethal alleles of Khc. Chromosomes mutagenized with ethylmethane sulfonate were tested over Khc, a null mutation. Those that failed to complement Khc were tested over Dif(2R)Jp6, a deletion that removes Khc. They were then tested over Dif(2R)Jp6 with a wild-type Khc transgene present in the background. Chromosomes that failed to complement Dif(2R)Jp6 and were rescued by the Khc transgene were maintained in balanced stocks for subsequent tests.

To determine the lethal profiles caused by the Khc mutations reported here, adult males and virgin females of the genotypes Khc/T (2, 3) CyO TM6B, Tb HU, and Dif(2R)Jp6/T2;SCyO TM6B, Tb HU were mated. After hatching, second instar larvae of the genotype Khc; Dif(2R)Jp6 were isolated and then observed for lethality throughout development as described previously (36).

**Stability of Mutant KHCs—**Adult male flies that carried each Khc allele in the following genotype: Khc/Dif (2R)Jp6, P[wi+, pw1118P];+, were collected and stored at −80 °C. P[wi+, pw1118P] is a stable P-element insert that contains a wild-type copy of Khc cDNA fused to a c-myc epitope tag (37). High level expression is driven by a ubiquitin promoter. The Myc-KHC fusion protein completely rescues the lethality caused by Khc null mutations.

Total protein was extracted from male flies by homogenization in cold extraction buffer as described previously at a volume of 8 μl of extraction buffer/animal (38). The homogenates were clarified by centrifugation: 2 fly equivalents of each supernatant were run on a 5–10% SDS-polyacrylamide gradient gel and then transferred to nitrocellulose (38). The Myc-KHC fusion protein migrated more slowly than native KHCs in SDS-polyacrylamide gel electrophoresis, allowing comparison of their relative levels. Both KHCs were detected by incubation in a mouse monoclonal anti-Drosophila KHC antibody, Flyk-2, diluted 1:50 followed by incubation in an alkaline phosphatase-conjugated goat anti-mouse serum at 1:1000 (36). Sequencing of Khc Alleles—DNA was isolated from Khc/Dif(2R)Jp6 pre-lethal larvae using the QiAmp Tissue Kit. Overlapping ~300-base pair fragments of genomic DNA covering the entire coding sequence of Khc were amplified separately using the polymerase chain reaction. The polymerase chain reaction products were then purified using the Qiaquick gel extraction kit. DNA was sequenced using a Thermo Sequenase sequencing kit, and the reactions were run on an ABI automated sequencer. Both sense and antisense strands were sequenced and in areas that had changes both strands were sequenced.

---

2 D. J. Rose, K. M. Brendza, and W. M. Saxton, manuscript in preparation.
Expression and Purification of K401-BIO for Motility—The expression plasmid pEY4, used for motility assays, contains a Drosophila Khc cDNA that encodes the COOH-terminal 87 residues of Escherichia coli ATCC-39 selection primer (5K401-37 (57.2, 75 mM NaCl, 2 mM MgCl2, 1 mM EGTA, 1 mM DTT, 0.02 mM ATP)). The column was eluted with a 600-ml linear salt gradient (75–600 mM NaCl). The K401 protein eluted at approximately 100 mM NaCl. Fractions enriched in K401 were pooled and dialyzed against DEAE buffer (20 mM Tris-HCl, pH 7.8, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.02 mM ATP). The dialyzed solution was clarified by ultracentrifugation and passed over a 15-ml DEAE-Sephacel column equilibrated with DEAE buffer (20 mM Tris-HCl, pH 7.8, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.02 mM ATP). The column was eluted with a 200-ml linear salt gradient (50–400 mM KCl). The K401 protein eluted at approximately 80–100 mM KCl. Fractions enriched in K401 were pooled and concentrated by ultrafiltration (Amicon Centriprep 30) to ~5 ml. The concentrated K401 was then dialyzed twice against ATPase buffer (20 mM Hepes, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate, 1 mM DTT, 5% sucrose adjusted to pH 7.2 with KOH), clarified, and stored at ~8 °C. This procedure yielded 4–10 mg of highly purified K401 per 30 g of E. coli.

The protein concentration of the kinesin preparations was determined by the Bradford method (Bio-Rad Protein Assay with ovalbumin and IgG as standards). It was also measured spectrophotometrically at A280 and A260 to determine the concentration of the stoichiometrically bound ADP (40). In addition, the concentration of active kinesin was determined using the creatine kinase-coupled assay described previously (39). The active site concentration for each preparation varied from 90 to 99% of the protein concentration estimated by the Bradford method. For the experiments reported, the concentration of kinesin is based on the active site concentration of the preparation.

Mammalian Brain Tubulin and Microtubules for Motility and Kinetic Experiments—Bovine brain tubulin was purified as described previously (40). On the day of each experiment, an aliquot of tubulin was thawed, cycled, and then stabilized with 20 μM taxol.

Motility Assays—Microtubule-gliding assays were performed using K401-BIO preparations as described by Berlinear et al. (39) except that α-casein was used in all washes instead of bovine serum albumin. Samples were observed by video-enhanced differential interference microscopy using an Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 100 × Plan-Neofluar objective, a Hamamatsu C2400 series video camera, and an Argus-10 image processor (Hamamatsu Photonics, Hamamatsu City, Japan). Images were recorded on Maxell S-VHS tapes using Mitsubishi model BV-1000 Professional Video Cassette recorder. Microtubule movements were measured directly from the video monitor.

ATPase Assays—ATPase assays were performed at 25 °C in ATPase buffer (0.4 mM a-mannose, 0.8 μM taxol) according to following the hydrolysis of [32P]ATP as described previously (40). All concentrations are reported as final values after mixing. To initiate an ATPase reaction, a 5-μl aliquot of MT/K401 complex (0.5 μM K401, 0.25–20.0 μM tubulin, and 20 μM taxol) was mixed with 5 μl of substrate (0.01–2.0 mM MgATP and trace [32P]ATP). After incubation for times varying from 5 to 120 s, the reaction mixture was quenched by the addition of 10 μl of 4 N HCl followed by the addition of 20 μl of chloroform, and neutralization with 8.7 μl of 2 M Tris, 3 M NaOH (final pH 7–7.8). 1.5 μl of each quenched reaction was spotted on a polyethyleneimine-cellulose TLC plate, and then developed in 0.6 M KH2PO4 buffer at pH 3.4. Radiolabeled ATP and ADP were quantitated using a FUJIX Bio-imaging Analyzer Bas 1000 with MacBS version 2.4 (Fuji Photo Film Co.; Kohshin Graphic Systems, Inc.).

The concentration of product ([α-32P]Pi) was plotted as a function of time, and the data were fit to a line. The rate of ATP hydrolysis was determined from the slope and then plotted as a function of either ATP or microtubule concentration. The fit of the data to a hyperbola (Kaleidagraph, Synergy Software, Reading, PA) provided the steady-state parameters: \(k_{cat} = \frac{V}{[ATP]}\), \(K_{M,ATP}\), and \(K_{M,MT}\).

The concentration of kinesin used in the steady state ATPase assays was 0.5 μM. This concentration was selected to ensure that kinesin motors were dimeric under the experimental conditions of the assay (\(K_d\) for dimerization ~40 nM (42)). The data in Fig. 7A were fit to a quadratic equation because the steady-state ATPase assays as a function of microtubule concentration were performed with tubulin concentrations that were as low as the enzyme concentration.
Mutations in Kinesin Motor Domain

| Khc allele number | Base changea | Percent of total gene sequenced | Codon changeb | Location in secondary structurec | Stable protein | Conservationd | Phenotype |
|------------------|--------------|---------------------------------|---------------|---------------------------------|----------------|---------------|-----------|
| 24               | C513T        | 96%                             | Q65STOP       | \(\alpha_1\)                     | No             | Severe        |
| 27               | C513T        | 96%                             | Q65STOP       | \(\alpha_1\)                     | No             | Severe        |
| 18               | G565A        | 77%                             | V80I          | \(\alpha_1\)                     | No             | CK            |
| 32               | G720C        | 98%                             | E134K         | \(\beta_4\)                      | Yes            | CK            |
| 23c              | G817T        | 99%                             | E164K         | \(\beta_5\)                      | Yes            | CK            |
| 19               | G856A        | 95%                             | E177K         | \(\beta_{5a/L8b}\)               | Yes            | IK            |
| 8                | C955T        | 95%                             | E218STOP      | \(\alpha_{3a}\)                  | No             | IK            |
| 17e              | C1064T       | 100%                            | S246F         | L11                             | Yes            | CS            |
| 2                | C1078T       | 96%                             | E251K         | L11                             | Yes            | NC            |
| 37c              | C1156T       | 97%                             | D277N         | \(\alpha_4\)                     | Yes            | CS            |
| 4                | C1198T       | 100%                            | T281M         | \(\alpha_5\)                     | Yes            | IS            |
| 36               | G1234A       | 89%                             | B303C         | \(\beta_8\)                      | Yes            | CS            |
| 10               | T1294A       | 95%                             | T323S         | \(\alpha_6\)                     | Yes            | CS            |
| 10               | C1396T       | 95%                             | E357K         | \(\alpha_7\)                     | Yes            | IK            |

a Base and amino acid numbering is according to Yang et al. (61).

b Secondary structural features are according to Kull et al. (26) and Kozielski et al. (24).

c Conservation abbreviations as follows: IS is identical in over 95% of the kinesin superfamily; CS is conserved in over 70% of the superfamily; IK is identical in 100% of the KHC subfamily; CK is conserved in 100% of the KHC subfamily; and NC is less than 100% conserved in the KHC subfamily.

d Mutations chosen for in-depth biochemical analysis.

RESULTS

Identification of Mutations in the Coding Sequence of the KHC—To identify amino acid changes that alter the mechanochemical functions of kinesin in vivo, we screened for recessive lethal mutations in the KHC gene of Drosophila. Forty independent mutant lines were established and changes in their KHC genes were identified through DNA sequence analysis. It has been shown that the NH2-terminal \(-400\) amino acids of KHC are sufficient for microtubule-based movement (43, 44). This abbreviated KHC can be truncated further to the NH2-terminal \(-350\) amino acids and still function as a microtubule-activated ATPase and motor although processivity is compromised and ATPase characteristics are altered (12, 41, 45–47). Therefore, we searched for missense changes in the NH2-terminal \(-350\) codons of the 40 mutant genes. Motor domain changes were found in 13 of them; 3 are nonsense and 10 are missense mutations (Table I). To address the possibility that other lesions in the 13 motor domain alleles contributed to the lethal phenotypes observed, sequencing was extended to the 3\(^{-}\) ends of their coding regions. One additional missense change was found in \(Khc^{29}\). Although the sequence analysis was not complete for all genes, it was extensive enough to show that the probability of any unidentified changes in a given motor domain allele is quite small (<0.02).

Stability of Mutant KHC Proteins in Vivo—The motor domain mutations were expected to have two different effects: some would change mechanochemical functions while others would reduce steady-state levels of KHC protein in vivo. To identify alleles that reduce steady state levels of protein, adult flies that carried a mutant \(\text{Khc}\) allele and a myc-tagged wild-type \(\text{Khc}\) allele were tested by Western blotting. The transgenic tagged allele overexpressed a functional KHC fusion protein that electrophoresed more slowly than native KHC, allowing visualization of the levels of the mutant KHCs (Fig. 2). The relative stabilities of all 13 of the motor domain alleles are shown in Table I. The three alleles with nonsense mutations (\(Khc^{24}, Khc^{27}\), and \(Khc^{30}\)) and one allele with a missense mutation (\(Khc^{18}\)) do not produce detectable KHC. The remaining 9 missense alleles produce roughly normal levels of KHC protein, suggesting that the mutant polypeptides can fold and accumulate in vivo. Therefore, the phenotypes caused by those 9 missense alleles are probably due to altered mechanochemical functions.

Selection of Alleles for in Vitro Characterization—Three criteria were considered in selecting a subset of the motor domain alleles for mechanochemical tests: the phenotypic severity caused by the allele, the evolutionary conservation of the affected residue, and the location of that residue in three-dimensional structural models of the KHC motor domain (24, 26, 27). To compare lethal phenotype severities, animals were genotyped to determine the location of the lethal allele on chromosome 2. If the mutation removes \(Khc\) from chromosome 2; \(m\)-\(Khc\), a fusion gene on chromosome 3 that expresses a Myc-tagged KHC at high levels; \(4, 17, 23, 27\), and \(37\), the respective mutant \(\text{Khc}\) alleles. The upper band corresponds to transgenic Myc-KHC whereas the lower band corresponds to endogenous wild-type or mutant KHC. The absence of detectable protein suggests that the nonsense allele \(\text{Khc}^{27}\) is a protein null. The 4 missense alleles that were selected for mechanochemical characterization (\(\text{Khc}^{4}, 17, 23, 37\)) all produce stable mutant KHCs.

![Fig. 2. Stability of mutant KHC proteins in vivo. A Western blot of cytosol preparations from adult flies of the indicated genotypes was probed with an antibody that binds to the motor domain of Drosophila (36). Genotypes: +, wild-type chromosomes; \(Df\), a deletion that removes \(Khc\) from chromosome 2; \(m\)-\(Khc\), a fusion gene on chromosome 3 that expresses a Myc-tagged KHC at high levels; \(4, 17, 23, 27\), and \(37\), the respective mutant \(\text{Khc}\) alleles. The upper band corresponds to transgenic Myc-KHC whereas the lower band corresponds to endogenous wild-type or mutant KHC. The absence of detectable protein suggests that the nonsense allele \(\text{Khc}^{27}\) is a protein null. The 4 missense alleles that were selected for mechanochemical characterization (\(\text{Khc}^{4}, 17, 23, 37\)) all produce stable mutant KHCs.](image)
Mutations in Kinesin Motor Domain

Fig. 3. Determination of the severities of KHC mutations in vivo. A graphic representation of the time course of lethality caused by mutant Khc alleles. The x axis shows successive stages in the development of Drosophila. The y axis shows the percent of live animals remaining at the beginning of each stage. Each line is labeled with an abbreviated genotype: numbers represent the different Khc alleles and Df represents a deletion of the Khc locus. The number of second instar larvae tested for each genotype were: Khc3/Df = 25, Khc4/Df = 122, Khc17/Df = 86, Khc17/Df = 86, Khc37/Df = 96, and Khc17/Df = 289. Note that the effect of Khc4 is similar to both the null mutation Khc37 (compare first and second lines) and the deletion (compare third and fourth lines). Therefore Khc4 causes a near complete loss of function and is classified as “severe” in Table I. Khc37 allows the survival of some animals to adulthood and thus is classified as “mild.” Khc23 and Khc17 are less severe than a null but do not allow survival of any adults and thus are classified as “moderate.”

affected in the motor domain alleles, sequence alignments and three-dimensional modeling were performed (Fig. 4 and Table I). Based on these data, four alleles covering the full range of in vitro severities were selected for in vitro characterization: Khc17, Khc23, Khc4, and Khc37. Khc17 is identical in all but one member of the superfamily and is located in a5 which is thought to be important for the microtubule-KHC interaction (22–24, 26, 27, 31, 48). Khc37 causes a mild lethal phenotype. The affected amino acid (Asp 277) is conserved in both the KHC subfamily and in the superfamily, frequently being substituted by a glutamic acid. It lies at the boundary of a4 and L12, which is also thought to be important for the microtubule-KHC interaction (31). Khc23 is a moderate allele and the affected residue (Glu164) is identical in the KHC subfamily as well as conserved in the superfamily. It is located at the boundary of b5a and L8 (b5a/L8b), which might participate in microtubule binding and in interactions between dimerized KHC motor domains (24, 31). Khc17 is another moderate allele and the affected residue (Ser246) is identical in the KHC subfamily except for the divergent KHC of Neurospora crassa which has a reasonably conserved substitution. It is located in L11 which might link changes in the structures of the ATP and the microtubule-binding sites (49, 50).

Microtubule Motility with Mutant KHC Proteins—To test the functional integrity of the four selected mutant KHCs in vitro, we first assayed their abilities to generate microtubule-based movement. Biotinylated, dimerized KHC motor domains (designated K401-BIO) were expressed in E. coli (39). To enrich for active motors, bacterial cytosol was subjected to a cycle of binding to microtubules in the presence of Mg/AMP-PNP and release with MgATP. The Western blot in Fig. 5 shows that this approach produced soluble KHC motor domains of the expected size. Each of the four mutant proteins was tested in parallel with wild-type K401-BIO at saturating MgATP concentrations (2.5 mM) in a microtubule-gliding assay developed by Berlinear et al. (39). All 5 types of K401-BIO motors could move microtubules. However, many microtubules were stationary and those that moved halted intermittently, even in the wild-type assays. This suggests that a significant fraction of the motors affixed to the coverslip were either inactive or in suboptimal orientations for full activity. Microtubule gliding velocities were measured from the fraction of microtubules that were motile during periods of smooth gliding (Table II). While the possibility of unperceived interruptions and variable drag forces hindered interpretation of the relative velocities, the fact that the mutant motors could indeed generate gliding movements indicated that each retained some mechanochemical capability.

Steady-state Kinetic Analysis of Mutant KHC Proteins—To gain more critical insights into the effects of the selected motor domain mutations on the KHC mechanochemical cycle, steady-state ATP turnover was analyzed. For those studies, we re-engineered motor domains containing only the NH2-terminal 401 amino acids of KHC (K401) without the biotin motility anchor. The new construct allowed purification of milligram amounts of highly active motor domains and facilitated comparison of our results to extensive previous kinetic studies of other KHC motor proteins. 31510

Fig. 4. Amino acid conservation and location of motor domain mutations selected for mechanochemical characterization. A, the amino acid changes caused by the four Drosophila motor domain alleles selected for mechanochemical characterization are placed above partial sequence alignments of Drosophila (Dm), human (Hs), and rat (Rn) KHCs, which are all members of the KHC subfamily, and Drosophila NCD, which is a member of the COOH-terminal motor subfamily (27, 58–61). The corresponding amino acid numbers for the different motor proteins are included to facilitate location of the affected amino acids in published motor domain crystal structures. B, location of the affected amino acids in a crystal structure model of the rat brain KHC motor domain (27). This model was rotated to display the locations of four wild-type amino acids that are changed in the motor domain alleles and the positions of their side chains. The helices and loops thought to be most directly involved in microtubule binding are on the left, and the nucleotide-binding pocket is at the rear near the top. The ADP in the active site is shaded yellow. The side chains of the wild-type residues altered in the motor domain mutations are green except for Khc17. The location of the residue affected by the Khc17 mutation cannot be shown accurately because loop 11 has not been resolved in KHC crystal structures. In the linear sequence of loop 11, the affected amino acid is 3 residues from the switch II region and 17 residues from the beginning of A4.

Two series of steady-state ATPase assays were performed on
the purified K401 proteins. Because KHC is a microtubule-activated ATPase (52), we first measured the rate of ATP hydrolysis as a function of microtubule concentration in the presence of saturating levels of MgATP (2 mM MgATP; Fig. 7A). In the second set of experiments, we measured the rate of ATP hydrolysis as a function of MgATP concentration in the presence of saturating levels of microtubules (18 μM tubulin; Fig. 7B). The mutant kinesins all exhibited Michaelis-Menten kinetics, and the steady-state kinetic parameters were determined: $k_{\text{cat}}$, $K_{\text{m,ATP}}$, $K_{\text{cat}}/K_{\text{m,ATP}}$, and $k_{\text{cat}}K_{\text{m,ATP}}$ (Table III).

The mutation that caused the mildest phenotype in vivo, Khc37, caused only slight changes in K401 kinetics while the mutations that caused moderate or severe phenotypes in vivo caused more substantial changes in kinetics. Tests of K401–37 revealed a $k_{\text{cat}}$ of 15 s$^{-1}$, suggesting that the ATPase cycle is somewhat slower than wild-type (20 s$^{-1}$). The $K_{\text{m,ATP}}$ (60 μM) was lower than wild-type (96 μM), suggesting somewhat tighter binding of ATP. The $K_{\text{cat}}/K_{\text{m,ATP}}$ at 0.5 μM was equivalent to wild-type (0.8 μM), suggesting normal interactions with microtubules. The $k_{\text{cat}}/K_{\text{m,ATP}}$ at 22 μM$^{-1}$ s$^{-1}$ (wild-type = 25 μM$^{-1}$ s$^{-1}$) supports this interpretation. The amino acid substitution in K401-37 (D277N) occurs at the junction of α4/L12 which we expect to be important in the mecanochanical cycle. Further insight into the role of Asp277 will require tests of other amino acid substitutions.

Tests of K401-4, the most severe allele in vivo, revealed that
KHC has ADP bound tightly in the catalytic pocket (13). Proper interaction with a microtubule causes release of the ADP which allows ATP access to the binding site (Fig. 1). Therefore, the weakened ATP binding of K401-4 could be a secondary effect of the altered microtubule-motor interaction. To determine if the mutation has a primary effect on ATP binding, we measured rates of steady-state ATP hydrolysis in the absence of microtubules. This “basal ATPase” rate for K401-4 (0.14 ± 0.005 s⁻¹) was 14-fold greater than for wild-type (0.01 ± 0.002 s⁻¹), suggesting that the T291M mutation does have an effect on the structure and function of the ATP-binding pocket that is independent of defects caused by aberrant microtubule binding.

Thr²⁹¹ is located in α5, which is connected to α4 by L12, on the microtubule-binding face of the motor domain. Previous work suggests that α5 and L12 are involved directly in microtubule binding (22, 24, 26, 27, 31, 48). Furthermore, the adjacent α4 via L11 is thought to be important in transmitting structural changes between the microtubule and ATP-binding sites, changes that link the ATPase and microtubule binding cycles (49, 50, 53). In current crystal structure models of KHC, the side chain of Thr²⁹¹ extends roughly parallel to the motor domain surface from α5 toward α4 (Fig. 4B). Substitution by the methionine side chain, which is longer, more hydrophobic, and lacks hydrogen bonding capacity, probably shifts the orientations of α5, α4, and L12. Such a structural shift could raise the basal ATPase rate by partially mimicking a conformational change that is normally induced by microtubule binding; a change that facilitates ADP-ATP exchange.

The results of the steady-state analysis of K401-17 indicate a significantly slowed ATPase cycle with a decrease in $k_{cat}$ from 20 s⁻¹ to 6 s⁻¹. The $K_{m,ATP}$ was 1.6 μM, a 2-fold increase but still a fairly tight microtubule-motor interaction. It is unlikely that weakened microtubule binding is responsible for the slow turnover because even high concentrations of microtubules (>15 μM tubulin) did not increase the turnover rate (Fig. 7A). Furthermore, it is unlikely that defective ATP binding is responsible for the slow turnover because neither the $K_{m,ATP}$ nor the basal ATPase rate (0.01 ± 0.003 s⁻¹) differed significantly from wild-type. The S246F amino acid change occurs in L11 which connects α4 on the microtubule-binding surface to the switch II region of the ATP pocket. As mentioned above, it is thought that L11/α4 could link conformational changes at the microtubule-binding interface to conformational changes in the catalytic pocket that are required for efficient turnover. The S246F substitution in K401-17 could slow the ATPase cycle by distorting that linkage.

K401-23 also has significantly slowed ATP turnover as shown by the 2-fold reduction in $k_{cat}$ to 10 s⁻¹. The $K_{m,ATP}$ of 0.7 μM was similar to wild-type (0.8 μM), suggesting that microtubule-motor binding is normal, but the $K_{m,ATP}$ was reduced 3-fold to ~30 μM, indicating that motor-ATP binding is abnormally strong. We expect that a change in the nucleotide pocket that tightens ATP binding also slows product release which in turn slows ATP turnover, a model consistent with product release limiting the ATPase cycle (13, 54, 55). This interpretation of the data suggests a change in the structure of the ATP-binding site, but the mutation in K401-23 (E164K) occurs at the βα/L8b junction, which is remote from the ATP-binding site (Fig. 4B). Current crystal structures and protease sensitivity studies agree that the 164 position is solvent-exposed throughout the mechnanochemical cycle (24, 26, 27, 56). Thus, it is reasonable to propose that the negatively charged Glu¹⁶⁴ is involved in an electrostatic interaction on the surface of the motor domain that has long-range effects on the structure of the nucleotide-binding site.

**DISCUSSION**

To probe the mechnanochemical mechanisms employed by kinesin we used random mutagenesis of *Drosophila* and DNA sequencing to identify amino acid changes in KHC that impair motor domain function *in vivo*. Of the 40 recessive lethal mutations studied, 10 have missense amino acid changes in the motor domain. Four of those were selected for further characterization. The 4 mutations range from a severe allele that causes a near complete loss of function to a mild allele that causes only a partial loss of function, as assessed, comparison of the lethal phenotypes that they cause with those caused by a null allele. After expression and purification of the motor domains fused to a biotin motility anchor, microtubule gliding assays showed that the mutant motors retained some mechnanochemical functions. However, the relative rates of gliding did not agree well with the relative severities of the *in vivo* phenotypes. We suspect that gliding rates are extremely sensitive to the presence of inactive motors that may interfere with microtubule gliding as well as the orientation of the active motors on the slide. Because of the combined effects of these two phenomena, the rates measured may not reflect accurately the mechnanochemistry of the active majority. Using a different motor domain construct without the biotin anchor and optimized for purification of active proteins, steady-state kinetic analysis of the mutant proteins revealed defects whose relative severities generally correlated with the relative severities of the *in vitro* phenotypes. These results suggest that the changes in steady-state kinetics of ATP turnover that we measured *in vitro* are physiologically relevant.

To interpret the kinetic effects of the mutations, we considered the evolutionary conservation of the amino acids that were changed and their positions in the atomic structure of a motor domain dimer. The allele that was mildest in *in vivo*, *Khc*³⁷, has an aspartic acid to asparagine change at the junction of α4 and L12. Asparagine is less polar than aspartic acid but their side chains are very similar in size. The steady-state kinetic analysis showed relatively mild effects, however, a change of the corresponding human KHC residue to alanine (nonpolar and small) caused a 2-fold reduction in $K_{m,ATP}$ (31). Combined, these results indicate that both the polarity and the size of this residue are important for correct microtubule-KHC interaction. Furthermore, since *Khc*³⁷ clearly causes axonal transport de-
fecteds (not shown) and semilethality, our results suggest that even slight changes in kinetics can have significant consequences in vivo.

The next mildest allele, Khc<sup>17</sup>, changes a serine to a phenylalanine in L11. Because L11 has not been resolved by crystallography, the position and orientation of the serine side chain is difficult to predict. However, one can assume that the change disrupts any interactions that the serine normally has because the phenylalanine side chain is dramatically larger and more hydrophobic. The S246F mutation causes a substantial decrease in \( k_{\text{cat}} \) (5.9 versus 20 s<sup>-1</sup> for wild-type), \( k_{\text{cat}}/K_{M,\text{ATP}} \) (0.065 versus 0.2 \( \mu \)M<sup>-1</sup> s<sup>-1</sup>), and \( k_{\text{cat}}/K_{M,\text{MT}} \) (3.6 versus 25 \( \mu \)M<sup>-1</sup> s<sup>-1</sup>), yet both the \( K_{M,\text{ATP}} \) and \( K_{M,\text{MT}} \) are close to wild-type. These results suggest that Khc<sup>17</sup> binds ATP and the microtubule lattice relatively normally, yet a key step important for ATP turnover is defective. This interpretation is consistent with the hypothesis that L11 acts as a structural link to couple microtubule binding to activation of the hydrolytic cycle (25, 53).

The most severe motor domain allele, Khc<sup>4</sup>, changes a threonine in a5 to a methionine (T291M). The methionine side chain is larger than that of threonine and more hydrophobic. Furthermore, the threonine side chain should form a hydrogen bond, perhaps with an amino acid in a4, and the methionine side chain would not hydrogen bond. Thus, the T291M mutation probably changes the orientations of a5, a4, and L12, which in turn cause shifts in other structural elements. The kinetic changes caused by the mutation suggest that the structures of both the ATP-binding pocket and the microtubule-binding site are altered to mimic a partially activated site. Consistent with this interpretation is the observation that there is a substantial increase in the rate of steady-state ATP turnover in the absence of microtubules. Our results and the fact that Thr<sup>291</sup> is invariant in the kinesin superfamily suggest that it has a key role in the transmission of structural changes between the microtubule- and ATP-binding sites.

The Khc<sup>23</sup> allele, less severe than Khc<sup>4</sup> and more severe than Khc<sup>17</sup>, changes a negatively charged glutamic acid to a positively charged lysine (E164K) at the β5a/L8b junction. The glutamic acid side chain is solvent exposed and remote from the ATP-binding pocket, yet the change to lysine causes tighter ATP binding and a 2-fold reduction in ATP turnover rate. A change of the corresponding glutamic acid in human KHC to an uncharged alanine also causes a 2-fold reduction in ATP turnover rate (31). Thus, it may be the loss of the negative charge on Khc<sup>23</sup> rather than the gain of the positive charge that causes the slow ATP turnover. Combined, these observations suggest that Glu<sup>164</sup> normally participates in an ionic interaction that influences the structure of the nucleotide-binding pocket during the ATPase cycle.

Our examination of KHC crystal structures revealed a possible mechanism for the influence of Glu<sup>164</sup> on nucleotide binding. The elongated, negative Glu<sup>164</sup> side chain extends away from β5a/L8b and the dimer interface toward the center of the microtubule-binding surface. It comes into close proximity with the elongated, positive side chain of Arg<sup>292</sup>, which projects from helix a5 toward β5a/L8b (Fig. 8). Given their proximity and opposite charges, Glu<sup>164</sup> and Arg<sup>292</sup> could interact to form an ionic link between a5 and β5a/L8b. It is worth noting that in human KHC, replacement of the Arg<sup>292</sup> equivalent by an uncharged alanine (R284A) (31) like our E164K change, causes a 2-fold reduction in ATP-turnover rate. It is also noteworthy that this Glu<sup>164</sup>/Arg<sup>292</sup> amino acid pair is highly conserved in the KHC and several other NH<sub>2</sub>-terminal kinesin subfamilies, but is very divergent in the COOH-terminal kinesin subfamily. We propose that a Glu<sup>164</sup>/Arg<sup>292</sup> ionic bridge coordinates the positions of L8 and a5, and that the effect of the E164K mutation on ATP binding is due to a misorientation of a5, that as discussed above for T291M, can influence the structure of the nucleotide-binding pocket.

It is interesting to consider the possibility that the β5a/L8b to a5 bridge is one part of an extended chain that links the nucleotide-binding pockets of dimerized KHCs. Kozielski et al. (24) pointed out the possibility of an ionic interaction between positive residues in L8b of one motor domain and negative residues in L10 of the partner motor domain.
would be transient because it would need to break during the mechanoochemical cycle to allow simultaneous binding of both motor domains to the microtubule and then would presumably reform in the opposite orientation. Kozlowski et al. (24) also noted that strand 7 of the central β-sheet links L10 directly to the switch II element of the nucleotide-binding pocket. Thus, a linkage extending from the switch II region of one head to the switch II element of the nucleotide-binding pocket. A lack of sequence conservation in L10 of KHCs from different species casts some doubt on this L8b-L10 linkage. However, the linkage of L8b to some part of the partner motor domain remains an attractive possibility for coupling dimer interactions to nucleotide and perhaps microtubule binding.

In summary, we have identified 9 amino acid residues that are critical for kinesin function in Drosophila. The results of kinetic analysis of lethal amino acid substitutions at some of those sites have revealed that the activity of the nucleotide-binding pocket is altered by structural changes on the side of the KHC that binds microtubules. The results also support the idea that structural elements distant from one another within a motor domain and between dimerized motor domains are tightly integrated such that defects in ATP turnover affect microtubule binding and defects in microtubule binding affect ATP turnover. The tight coupling of these activities is not surprising when one considers the demands placed on kinesin in vivo. Small organelles can use only a few kinesin molecules to sustain processive transport, and missteps are costly, as illustrated by the effects of even mild kinesin mutations on axonal transport. It appears that a few stalled axonal organelles can trigger massive traffic jams in Drosophila axons that cause serious declines in neuron function (34, 57). Mechanistic studies of mutated KHCs are in progress to evaluate further the kinetic-structural relationships that underlie the precise coordination required for processive movement.

Acknowledgments—We thank Jeff Gelles for providing the K401-BIO construct, Lisa Engler for assistance with molecular modeling, and members of the Gilbert laboratory for assistance with protein purification and kinetics. We acknowledge Kathy Regan, Luke Dunlap, Shannon Kernick, Angel “Spike” Martindale, Jackie “Hammer” Armbrust, Jennifer Walcot, Scott Beason, and Kathy Sheehan for finding new KHC mutants. We also thank Elizabeth Raff, Ivan Rayment, and members of the Saxton laboratory for stimulating discussions, and Joe Howard and Smita Patel for critical reading of the manuscript.

REFERENCES

1. Hirokawa, N. (1998) Cell 94, 945–951
2. Hirokawa, N. (1996) EMBO J. 15, 5315–5320
3. Sack, S., Muller, J., Marx, A., Mandelkow, E.-M., and Sack, S. (1990) J. Cell Biol. 119, 383–389
4. Kuznetsov, S. A., and Gelfand, V. I. (1986) FEBS Lett. 217, 1–5
5. Ma, Y.-Z., and Taylor, E. W. (1997) J. Biol. Chem. 272, 742–750
6. Ma, Y.-Z., and Taylor, E. W. (1997) J. Biol. Chem. 272, 742–750
7. Ma, Y.-Z., and Taylor, E. W. (1997) J. Biol. Chem. 272, 742–750
8. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
9. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
10. Ma, Y.-Z., and Taylor, E. W. (1997) Biochemistry 36, 792–799
11. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
12. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
13. Ma, Y.-Z., and Taylor, E. W. (1997) Biochemistry 36, 792–799
14. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
15. Ma, Y.-Z., and Taylor, E. W. (1997) Biochemistry 36, 792–799
16. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
17. Ma, Y.-Z., and Taylor, E. W. (1997) Biochemistry 36, 792–799
18. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689
19. Ma, Y.-Z., and Taylor, E. W. (1997) Biochemistry 36, 792–799
20. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6685–6689