Kinetic Effects of Kinesin Switch I and Switch II Mutations*

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We have examined several mutants in the switch I, switch II region of rat kinesin. Pre-steady-state kinetic analysis of association and dissociation of an N256K mutant with nucleotides and microtubules demonstrates that the mutation blocks microtubule stimulation of nucleotide release and ATP hydrolysis without affecting other kinetic parameters. The results suggest that ADP release on one head may be coupled to structural changes on the other head to stimulate ATP hydrolysis. Mutations at Glu237, a residue predicted to participate in a hydrogen-bond interaction critical for nucleotide processing, reduced or abolished microtubule-dependent ATPase activity with only minor effects on pre-steady-state rates of nucleotide release or binding. Mutations at Glu200, a residue that could serve as an alternate electron acceptor in the above-mentioned hydrogen-bond interaction, had small effects on microtubule-dependent ATPase activity despite modestly reducing the rate at which microtubule-stimulated nucleotide release occurs. These results further clarify the pathway of coupling of ATP hydrolysis to force production.

Gilbert and Johnson (1, 2) proposed a detailed alternating site kinetic model for the mechano-chemical cycle of the conventional kinesin motor, which outlines the manner in which ATP binding and hydrolysis are coordinated to the binding and release of kinesin heads on the microtubule surface. However, the structural and thermodynamic basis for this coupling remains to be resolved. Alternating weak and strong microtubule binding between trailing and leading motor domains are thought to lead to net movement. ATP binding and/or hydrolysis may induce conformational changes in the protein that produce strain, which is relieved by subsequent conformational changes resulting in an overall displacement of the motor along the microtubule (reviewed in Ref. 3). The nucleotide binding pocket, visible in the crystal structure (4), contains a γ-phosphate sensor apparatus, consisting of four domains that are conserved throughout the kinesin superfamily, as well as among the myosins and the signal transduction G-proteins (3, 5–7). Crystal structures of kinesins, as well as structurally related domains of myosins and G-proteins, reveal distinct conformational states depending on whether the active sites are occupied by ADP or an ATP analog (3, 5, 6, 8, 9), and EPR spectroscopy of spin-labeled motors supports a multiple-state hypothesis (10, 11). Fundamental questions remain to be answered regarding the manner by which structural changes at the active site are coupled to interactions at the microtubule binding site to produce a force for movement.

A mutation that appeared to decouple the nucleotide-processing and microtubule-binding functions of the kinesin motor domain was identified in the minus-end-directed kinesin-like motor Kar3 (12, 13). Alignment of the Kar3 and rat conventional kinesin sequences identifies the residue Asn256 in the latter sequence as corresponding to Kar3 Asn256. In the crystal structure of rat conventional kinesin (4), the position of Asn256 defines the boundary between helix α4 and loop L11 (Fig. 1), although the loop is not resolved in the crystal structure. Here we address the questions of whether the mutation N256K in a processive, plus-end-directed kinesin would yield similar effects as did N650K in Kar3, and whether those effects could be localized within the kinetic pathway of conventional kinesin as outlined in the Gilbert-Johnson alternating site model (2).

Two additional sites, switch I (consensus sequence NXXYSSR, residues 199–204 in rat conventional kinesin) and switch II (consensus sequence DXXGXE, residues 232–237 in rat conventional kinesin), forming part of the catalytic core of kinesin (3, 8), served as additional targets for mutagenesis, the consequences of which are described in this report. The examination of crystal structures has suggested the existence of a phosphate sensor apparatus that includes switches I and II in the motor domains of kinesin, myosin, and the G-proteins. The structures participate in a switch mechanism, able to adopt two conformations depending on which nucleotide occupies the binding site. A third motif, the P-loop, stabilizes the α- and β-phosphates of the bound nucleotide. In myosin, a salt bridge between the conserved switch I arginine and switch II glutamate appears in the ATP-like state, in co-crystals of myosin and ATP analogs (14, 15). In the nucleotide-free and ADP-states the salt bridge is broken (16, 17). Furthermore, the nucleotide binding pockets of myosin, EF-Tu, and p21<sup>ss</sup>, all of which utilize a switch motif similar to that of the kinesins, appear to undergo a conformational change in response to ATP hydrolysis that moves the conserved switch II glycine toward the conserved P-loop glycine. The ADP state is considered “open,” whereas the ATP state is “closed,” because the binding pocket appears to contract around the bound nucleotide in the closed state (7).

Rat conventional kinesin has been crystallized in the ADP state only (4), and among the structures determined for kinesin-like proteins, little evidence of a two-state phosphate sensor switching mechanism can be found despite the presence of the conserved switch I and switch II sequences. The conformations of the nucleotide binding pockets of the two subunits are distinguishable, despite both being bound to ADP. The conformation of the switch domain in one subunit, in which a salt bridge between Arg<sup>204</sup> and Glu<sup>237</sup> is visible, resembles that of the myosin-closed state, visible in co-crystals of myosin and ATP analogs (14, 16). In the other subunit, however, hydrogen bonding between Arg<sup>204</sup> and Glu<sup>200</sup> appears possible, if only transitory. This observation suggests that it may be of some importance to the mechanism of kinesin, and that the side chain of Arg<sup>204</sup> may toggle between hydrogen bond interactions with Glu<sup>200</sup> on switch I and Glu<sup>237</sup> on switch II (Fig. 1).

Analyses of kinesins containing salt-bridge mutations reveal defects in the steady-state parameter <i>k</i><sub>cat</sub> (11, 18, 19). A <i>Drosophila</i> switch I R210A mutant was found to be defective not only in ATP hydrolysis, but microtubule detachment as well, with ATP binding and microtubule association unaffected (19). The R210K mutant is similarly impaired in...
ATPase function, but microtubule detachment is restored (20). Several studies of kinesin and myosin active sites have suggested that the salt bridge formed between switch I and switch II residues is critical in stabilizing a water molecule required in the catalysis of the ATPase reaction (21–24).

The experiments described here concern mutations engineered into the phosphate sensor apparatus of KHC407A. The mutations E200A and E200D were intended to disrupt any possible electrostatic interaction between switch I glutamate 200 and switch II arginine 204. The E200D mutation deletes a methylene group from the acidic residue but preserves the overall charge distribution of the system, whereas E200A removes the acidic residue altogether. A similar rationale was used in selecting the E237A and E237D mutations. It is important to note that only Glu237, a well conserved residue, corresponds to that residue in the myosin motor domain that forms a salt bridge with the switch II arginine; whereas, Glu200 is not part of the canonical switch I sequence. Another similarity is that E200 is located in a negatively charged region that forms a salt bridge with the switch II arginine, whereas, Glu237 is located in a positively charged region.

Finally, the effects of an N256K mutation, corresponding to N650K identified in the yeast kar3 gene, on the transient-state kinetics of the rat kinesin (0.0062 time constant) was determined by active-site titration based on [32P]ATP, whereas the second is independent of ATP, step b describes the binding of kinesin (containing an ADP at each site) to the microtubule surface followed by the release of one ADP (step c) to form the "captive head state." This sequence represents the pathway for kinesin to bind to the microtubule and enter the processive ATPase cycle. In the alternating ATPase cycle two ADP release events occur after kinesin binds to microtubules; the first is independent of ATP, whereas the second is stimulated by ATP (2, 29, 30).

RESULTS

ATPase Model—Throughout this report we will refer to Fig. 2 showing the Gilbert-Johnson alternating site ATPase pathway expanded to include steps involving ADP release that are not part of the normal cycle. In the alternating ATPase cycle two ADP release events occur after kinesin binds to microtubules; the first is independent of ATP, whereas the second is stimulated by ATP (2, 29, 30). The concentration of each kinesin preparation was determined by active-site titration based on measurement of the extent of [α-32P]ADP release from the enzyme-nucleotide complex as a function of time using methods described in the accompanying report (25). For the mutants E200A, E200D, and E237D, an active-site concentration was defined using this method. Rate constants governing ADP release in the absence of microtubules were measured for these three mutants were accelerated compared with that of the wild type kinesin (0.0062 ± 0.0003 s⁻¹); 0.0096 ± 0.0001 s⁻¹ for E200D, 0.010 ± 0.0005 s⁻¹ for E200A, and 0.013 ± 0.0003 s⁻¹ for E237D. A similar effect was observed for the N256K mutation, which gave an ADP release rate constant of 0.014 ± 0.001 s⁻¹. The E237A mutant was
unusual in that no estimation of active-site concentration was possible using this method. Direct measurements of mantADP release indicated a release rate of \( \sim 3 \text{ s}^{-1} \) after rapid mixing with ATP (in the absence of microtubules), indicating that the ADP dissociation rate constant for the E237A variant must be substantially (\( \sim 500\)-fold) accelerated over that of the wild-type motor. This rate was too fast to be resolved in our active-site titration experiment involving manual mixing. It is noteworthy that for each mutant and wild-type the release of ADP (steps d and e) of Fig. 5. The inclusion of steps d and e, the slow microtubule-independent release of ADP, were required for the analysis of mantADP release from the N256K variant (see Fig. 5D). Note that the “captive head state” is defined by the species, which is identified in the figure as M-K\(_{ADP}^i\).

### TABLE ONE

| Primers used for mutagenesis | Sequence                                      |
|------------------------------|-----------------------------------------------|
| N256K                        | 5'-GATGAAGCTAAAAATATCAAAAAAGTCTTTGCTTGCTTTGG-3' |
|                              | 5'-CCAAGACGAGCAGACAAAAAGCTTTTTGATATTTTACGGTCATC-3' |
| E200A                        | 5'-GCACAAATGACGACGATCGAGCCTTTGCTTGCTTTGCT-3'    |
|                              | 5'-CCAAGACGAGCAGACGATCGAGCCTTTGCTTGCTTTGCT-3'    |
| E237A                        | 5'-GATTTGCTGGGAGTCTGACGAGCAAAACTGG-3'           |
|                              | 5'-CCCAGTTTTGCTGACCTTTGCACTCCCAGCAAAATTGCT-3'    |
| E237D                        | 5'-GATTTGCTGGGAGTCTGACGAGCAAAACTGG-3'           |
|                              | 5'-CCCAGTTTTGCTGACCTTTGCACTCCCAGCAAAATTGCT-3'    |

**FIGURE 2.** Reaction scheme used to simulate mantADP release from kinesin-microtubule complex. The Gilbert-Johnson alternative site mechano-chemical pathway (2) is depicted along with additional ADP dissociation steps (c, d, and e) required during simulation of fluorescence data for the analysis of mantADP release results shown in Fig. 5. The inclusion of steps d and e, the slow microtubule-independent release of ADP, were required for the analysis of mantADP release from the N256K variant (see Fig. 5D). Note that the “captive head state” is defined by the species, which is identified in the figure as M-K\(_{ADP}^i\).
Steady-state activities of mutants

Initial rate of [α-32P]ATP hydrolysis was determined for each kinesin variant. Initial reaction conditions were 0.05 μM kinesin, 1 mM [α-32P]ATP, and 80 μM microtubules in ATPase buffer. Acid-quenched reactions were analyzed for progress of hydrolysis by thin layer chromatography, and initial hydrolysis rates are expressed as kcat.

|          | kcat/s⁻¹ |
|----------|----------|
| w.t.*    | 40       |
| E200A    | 28.8     |
| E200D    | 23.8     |
| E237A    | <0.1     |
| E237D    | 4.4      |
| N256K    | 0.3      |

*Wild-type data are from the accompanying report (25).

follow a single exponential, suggesting that the two sites are independent and identical in the absence of microtubules.

Steady-state ATPase Activity of KHC407A Mutants—Values for steady-state ATPase kcat were determined for the kinesin mutants at near saturating concentrations of microtubules (80 μM) and ATP (1 mM) and are summarized in TABLE TWO. It is apparent that ATP hydrolysis activity is more sensitive to mutations at residue Glu237 than at Glu200. This was expected, because the conserved Glu237 residue is believed to play a direct role in the catalytic mechanism of ATP hydrolysis by myosin, and the GTPases p21ras and EF-Tu (24, 31–33). The conservative replacement of this residue with an aspartate results in a 10-fold decrease in ATPase activity, whereas the elimination of the functional group by alanine substitution reduces ATPase activity to about 1/4 of the maximum achievable rate of ATP hydrolysis for wild-type kinesin, as well as by myosin subfragment S1, with kinetics resembling those observed for the natural substrate (30, 34). The concentration dependence of the rate of mantATP binding to N256K, E200D, and E237D variants of kinesin bound to microtubules were measured in the stopped-flow apparatus. In the microtubule-kinesin complex, one ADP is lost in forming the captive head state, so one site is free to bind ATP (see Fig. 2). The nucleotide derivative, mantATP, has been shown to bind to and be hydrolyzed by an N-terminal fragment of Drosophila and rat conventional kinesin, as well as by myosin subfragment S1, with kinetics resembling those observed for the natural substrate (30, 34). The concentration dependence of the rate of mantATP binding for N256K, E200D, and E237D is shown in Fig. 3. Fig. 3A shows representative data fit to a single exponential to determine the rate. In Fig. 3B, the rate data for each experiment were fit to a hyperbola by nonlinear regression, and kinetic parameters kmax, Kd, and kmax/Kd of the concentration of mantATP at which kobs is half of kmax were extracted (TABLE THREE). The initial slope of the plot, calculated by kmax/Kd, provides lower limit of the second order rate constant governing the binding of mantATP to the kinesin-microtubule complex (step 1 of the cycle).

The binding of mantATP to the kinesin-microtubule complex is not altered by more than 2-fold in either direction by any of the mutations tested. The mutation E237D, which may disrupt the salt bridge believed to form between the altered residue on switch II and Asp204 on switch I, shows the slowest rate of binding, with an apparent second order rate constant of 2.1 μM⁻¹ s⁻¹, half that of the wild-type motor. The E200D mutation, by contrast, binds mantATP with a rate constant of 11 μM⁻¹ s⁻¹, about twice that of the wild-type motor. Both the N256K and E237D mutations cause an increase in the maximum achievable rate of mantATP binding (nearly 400 s⁻¹) twice that of the wild-type motor. E200D, on the other hand, leaves kmax unchanged.

Microtubule Dependence of ADP Release from KHC407A Mutants—Upon rapid mixing with microtubules, the kinesin-mantADP complex will release mantADP according to a three-step pathway governed by steps a, b, and c (35, 36). At high microtubule concent-

![Figure 3. Binding of mantATP to KHC407A mutants. 10 μM tubulin and 2 μM kinesin mutants N256K (triangles), E237D (squares), and E200D (diamonds)](image)

|          | kmax/s⁻¹ | Kd/μM | kmax/Kd μM⁻¹ s⁻¹ |
|----------|----------|-------|------------------|
| w.t.*    | 210 ± 25  | 38 ± 10 | 5.6 ± 1.7       |
| E200D    | 190 ± 11  | 18 ± 3  | 11 ± 2           |
| E237D    | 396 ± 31  | 189 ± 22 | 2.1 ± 0.3      |
| N256K    | 384 ± 28  | 54 ± 6  | 7 ± 1            |

*Wild-type data are from the accompanying report (25).
trations, the observed rate of mantADP release ($k_{obs}$) is predicted to approach the value of the rate constant governing the release of the first mantADP (step b); a slower phase involving the release of the second mantADP (step c) can be seen only if unlabeled nucleotide is added to prevent the rebinding of mantADP. At low microtubule concentrations, the dependence of $k_{obs}$ on microtubule concentration provides an estimate of the rate constant governing kinesin-microtubule association (step a). In the accompanying report (25), $k_{obs}$ for mantADP release by wild-type rat conventional kinesin upon rapid mixing with microtubules failed to reach a maximum limit even at the highest microtubule concentration tested (80 μM), suggesting a large value for the rate constant governing nucleotide release (step b). In this report, we measure the rate of mantADP release by the kinesin variants N256K, E200D, and E237D upon rapid mixing with microtubules as shown in Fig. 4. The three mutant variants tested revealed values of $k_{obs}$ that were dependent on microtubule concentration according to a hyperbolic relationship in each case, best fit by a curve of the form $k_{obs} = k_{max}[Mt]/(M_{50} + [Mt])$, where $M_{50}$ is that concentration of microtubules yielding a $k_{obs}$ at half of $k_{max}$. The apparent rate constant for kinesin-microtubule association, equivalent to the initial slope of the curve, is calculated by the ratio of $k_{max}/M_{50}$ and the parameters obtained for the various mutants are compared with the wild-type motor in TABLE FOUR. Values for $k_{cat}$ reflected in the y-intercept of the curves, were not measurable for any of the mutants; in contrast, a value of 30 s$^{-1}$ was previously obtained for the dissociation rate $k_{off}$ of microtubules from the wild-type KHC407A.

The E200D mutation (Fig. 4A), with only a mild defect in $k_{cat}$, gave a 3-fold increase in the motor-microtubule binding rate, but a smaller and measurable mantADP release rate. The asymptotic approach of the fit curve for the E200D data is likely to reflect the behavior of the enzyme, whereas in the case of E237D, the data could have easily (but less accurately) been fit to a linear model (Fig. 4B). Both fits yield values of ~230 s$^{-1}$ for the maximum rate suggesting that the rate constant governing mantADP release from these mutants is lower than that of the wild-type motor by a factor of ~5. This is somewhat surprising in the case of E200D, whose steady-state rate of ATP hydrolysis is similar to that of the wild-type motor. However, this observation supports our conclusion that ADP release is not rate-limiting during steady-state turnover. The reductions in $k_{cat}$ for E200D and E237D are less dramatic and reflect the fact that ADP release is normally so much faster than the rate-limiting steps during steady state turnover so that a modest reduction in rate of ADP release is masked in the steady state.

The N256K mutation appears to reduce the rate of motor-microtubule binding to less than one-fifth that of wild-type (Fig. 4C), but the most significant effect is an estimated 800-fold reduction in the rate of mantADP release (step b) following microtubule binding. Although this step per se is not on the ATPase pathway during processive movement, it parallels step 2 of the cycle in that microtubule binding stimulates ADP release. It is clear from these results that the small $k_{cat}$ value seen for the N256K variant is due to a large reduction in the rate constant governing microtubule-stimulated mantADP release.

**ATP Dependence of mantADP Release from KHC407A Mutants—**

Motor-microtubule binding (step a) is followed by the rapid release of one bound ADP molecule (step b), whereas the rate of release of the second ADP is a function of ATP concentration (steps 1 and 2). In the accompanying report (25), KHC407A-mantADP complex exhibited a rapid release of

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**FIGURE 4. Microtubule dependence of ADP release from KHC407A mutants.** Fluorescence decay from kinesin-mantADP complex (1 μM kinesin, 2 μM mantADP) upon rapid mixing with microtubules (5–40 μM) plus ATP (1 mM) was measured. Fitting of a double-exponential curve to data gave fast- and slow-phase rates of fluorescence decay at each microtubule concentration. Fast-phase rates are plotted along with a best-fit hyperbola. A, E200D; B, E237D; C, N256K. Slow-phase rates were of low and variable amplitude relative to fast-phase rates and are omitted from the graphic and analyses. Kinetic parameters extracted from curve fitting summarized in TABLE FOUR.

**TABLE FOUR.** Microtubule dependence of mantADP release from KHC407A mutants

| Mutant  | $k_{max}$ (s$^{-1}$) | $k_{on}$ (μM$^{-1}$ s$^{-1}$) | $k_{off}$ (s$^{-1}$) |
|---------|---------------------|-----------------------------|---------------------|
| w.t.$^*$| ~1000               | 4.6 ± 0.1                   | 30 ± 3              |
| E200D   | 230 ± 12            | 13 ± 1.5                    |                     |
| E237D   | 226 ± 24            | 3.9 ± 0.8                   |                     |
| N256K   | 1.215 ± 0.005       | 0.70 ± 0.02                 |                     |

$^*$ Wild-type data are from the accompanying report (25).
its bound nucleotides upon being mixed with microtubules and ATP, with rate constants in excess of 1000 s\(^{-1}\). In the experiments described here, the rat conventional kinesin variants E200D, E237A, E237D, and N256K containing bound mantADP were mixed in the stopped-flow apparatus with microtubules plus various concentrations of ATP. These experiments are expected to reveal two fluorescence decay modes. The rate of the fast phase depends on microtubule concentration and is independent of ATP, and the rate of the slow phase is a function of ATP concentration. This experiment quantifies the most important kinetic parameters governing alternating site ATPase cycle in which ATP binding to one head stimulates the release of ADP from the other head.

In Fig. 5 are plotted the fluorescence traces representing mantADP release from KHC407A. Kinesin-mantADP complex (1 \(\mu\)M enzyme active site, 2 \(\mu\)M mantADP) was rapidly mixed with microtubules (16 \(\mu\)M) plus ADP, or ATP, and fluorescence decay monitored. Solid lines represent normalized fluorescence data. A, E200D; ADP (10 \(\mu\)M), ATP (1, 2, 4, 7.5, 12.5, and 22.5 \(\mu\)M). B, E237A; ADP (10 \(\mu\)M), ATP (2.5, 5, 10, and 15 \(\mu\)M). C, E237D; ADP (10 \(\mu\)M), ATP (2.5, 5, 10, and 15 \(\mu\)M). D, N256K; ATP (500 \(\mu\)M) in the absence of microtubules, ADP (10 \(\mu\)M), ATP (25, 50, 100, and 250 \(\mu\)M). Dashed lines are kinetic simulations of the reaction, based on a reaction depicted in Fig. 2, rate constants in TABLE FIVE, and initial reagent concentrations used in the experiments.

The fluorescence data for both E200D and E237D mutants fit by simulation of the reaction shown in Fig. 2, similar to that used for kinetic simulations for the wild-type enzyme, and rate constants generating best-fit curves (TABLE FOUR). The kinetics of the observed reaction are similar to those of the wild-type except that the rate constants governing mantADP release (steps b and 2) are at least an order of magnitude smaller than they are in the wild-type enzyme. By contrast, the rate constants for the E237D mutant reveal no obvious defect in the kinetics of the mutant. The E237A mutation resulted in a kinesin that could not be assayed by active-site titration, as discussed previously, because no bound \([\alpha-32P]ADP \) could be detected. The results of the mantADP release experiments described here suggest that a nucleotide-binding site is present in this mutant. They suggest that two mantADP release events are possible, and that the presence of ATP accelerates the second event, whereas the first is microtubule-dependent, similar to what was observed for Drosophila (2) as well as wild-type rat conventional kinesins described in the accompanying report (25).
Pre-steady-state Kinetics of ATP Hydrolysis by KHC407A Mutants—ATP hydrolysis by the N256K, E237A, and E237D mutants were measured using rapid quench analysis. Motor-microtubule complex (4 μM motor, 20 μM microtubules) was rapidly mixed with an equal volume of [α-32P]ATP (200 μM) and acid-quenched after intervals from 5 to 200 ms. None of these mutants showed detectable burst of ATP hydrolysis in 200 ms (data not shown). It is apparent from these results that the defects caused by each of the tested mutations causes a reduction in the rate of ATP hydrolysis in each mutant (Fig. 2, step 3). Because it is well established that microtubules stimulate the rate of ATP hydrolysis by kinesin, it appears that these mutations may interfere with the communication from the microtubule binding site of the motor to the ATPase site, which would otherwise accelerate catalysis.

**DISCUSSION**

Of the rat kinesin mutations tested, three (N256K, E237A, and E237D) were found to have substantial defects in steady-state ATP hydrolysis in the presence of microtubules, whereas mutations at the Glu200 residue had only marginal effects on $k_{cat}$. The effects of these mutations on the binding of mantATP were insufficient in all of the cases to account for overall steady-state defects. Nevertheless, effects on this step deserve some commentary. The results summarized in TABLE THREE show that the maximal rate of mantATP binding, for the mutants N256K and E237D is elevated ~2-fold over that of wild-type, despite disparate binding rate constants for the two mutants. Because the maximum observed rate represents the rate constant governing a first-order reaction occurring immediately after the formation of a “collision complex” between ATP and kinesin (1, 37, 38) the observed reaction may reflect a conformational change in the protein leading to the fluorescence enhancement. This step is not included explicitly in our model, but may be related to changes in structure preceding ATP hydrolysis. Both mutations also reduced steady-state ATP hydrolysis rates. The remaining mutation studied, E200D, reducing $k_{cat}$ less dramatically, has little effect on the maximum rate of the mantATP fluorescence change. Our results suggest that the nonconserved residue Glu200 in rat kinesin is of limited utility in the motility cycle of rat conventional kinesin.

The fluorescence change observed due to the release of mantADP from the N256K mutant motor upon binding to microtubules plus varying concentrations of ATP (Fig. 5D) were consistent with a model in which the rate constant governing mantADP release had been diminished ~500-fold compared with that of the wild-type enzyme. ATP binding appears to be unaffected, as indicated by the results of mant-ATP binding experiments (Fig. 3), as is microtubule binding, as indicated by mantADP release experiments (Fig. 4C). These results provide further mechanistic details to support the conclusions that N256K interferes with communication from the microtubule binding site to the nucleotide site, without altering the interactions with nucleotide or microtubules.

In the crystal structure of the dimeric rat kinesin, Asn256 is at the N terminus of α4, and defines the boundary between the helix and a disordered loop (4). The α4-L-11 domain appears to play a significant role in the communication of nucleotide phosphorylation state information to other domains within the motor, for example, the microtubule-binding region. Evidence for such a role for helix α4 is strongest in the crystal structures of myosin, crystallized in ADP, ATP-like, and nucleotide-free states (14, 15, 39). Finally, crystal structures of Kif1 in the ADP and ATP-like states suggest that hydrolysis of ATP is accompanied by a recruitment by helix α4 of ~10 residues from loop L11 (6). Residue Asn256 in rat conventional kinesin is solvent-exposed. The replacement of asparagine by lysine introduces a basic side chain in lieu of a neutral hydrophilic one. It is not clear from kinesin crystal structures what, if any, salt bridges that lysine may participate in at this position, nor is it yet possible to evaluate the contacts made by this residue with those on the microtubule surface. Nevertheless, it is clear from the results of these experiments that the N256K mutation in rat kinesin interferes with the communication from the microtubule binding site to the nucleotide binding site. The N256K mutation has a similar effect on the kinetics of the kinesin switch I and II mutants (step 1). Similarly, kinesin-microtubule binding rate appears to be unaffected (step a). The slow release of the hydrolysis product ADP by the mutant motor suggests that a mechanism for toggling the active site between “high affinity” and “low affinity” states has been impaired by the N256K mutation, such that bound nucleotide is released slowly regardless of whether it is in the triphosphate or diphosphate state. Although only the second ADP release (step 2) is dependent on ATP bind-

**TABLE FIVE**

| ATP dependence of ADP release from KHC407A mutants |
|------------------------------------------------------|
| Global fitting of KINSIM-simulated kinetic data to mantADP release data generated a set of kinetic parameters for best-fit curves depicted by dashed lines in Fig. 5. Step numbers correspond with numbered reaction steps in the pathway shown in Fig. 2. Forward ($k_+$) and reverse ($k_-$) rates are shown. |
| w.t. | $k_+$ | $k_-$ |
| a | 7.8 μM$^{-1}$ s$^{-1}$ | 9.3 s$^{-1}$ |
| b | >1000 s$^{-1}$ | 42.9 μM$^{-1}$ s$^{-1}$ |
| 1 | 1.7 μM$^{-1}$ s$^{-1}$ | 18.4 μM$^{-1}$ s$^{-1}$ |
| 2 | >1000 s$^{-1}$ | 33.2 μM$^{-1}$ s$^{-1}$ |
| c | 1 s$^{-1}$ | |
| E200D | | |
| a | 18 μM$^{-1}$ s$^{-1}$ | |
| b | 200 s$^{-1}$ | 9 μM$^{-1}$ s$^{-1}$ |
| 1 | 3.2 μM$^{-1}$ s$^{-1}$ | |
| 2 | 100 s$^{-1}$ | 11 μM$^{-1}$ s$^{-1}$ |
| c | 1.8 s$^{-1}$ | |
| E237A | | |
| a | 12 μM$^{-1}$ s$^{-1}$ | 50 s$^{-1}$ |
| b | 400 s$^{-1}$ | |
| 1 | 2 μM$^{-1}$ s$^{-1}$ | 10 s$^{-1}$ |
| 2 | 280 s$^{-1}$ | |
| c | 3.5 s$^{-1}$ | |
| E237D | | |
| a | 5.5 μM$^{-1}$ s$^{-1}$ | 60 s$^{-1}$ |
| b | >1000 s$^{-1}$ | 90 μM$^{-1}$ s$^{-1}$ |
| 1 | 2 μM$^{-1}$ s$^{-1}$ | 10 s$^{-1}$ |
| 2 | >1000 s$^{-1}$ | 2 μM$^{-1}$ s$^{-1}$ |
| c | 3.5 s$^{-1}$ | |
| N256K | | |
| a | 6.1 μM$^{-1}$ s$^{-1}$ | 7.0 s$^{-1}$ |
| b | 2.3 s$^{-1}$ | |
| 1 | 0.8 μM$^{-1}$ s$^{-1}$ | |
| 2 | 1.8 s$^{-1}$ | |
| c | 0.4 s$^{-1}$ | |
| d, e | 1 s$^{-1}$ | |

*Wild-type data are from the accompanying report (25).*
ing, both ADP release steps presumably require communication between the microtubule binding domain and the nucleotide binding site, and the mechanical coupling between the two is a candidate for that which is directly impaired by the N256K mutation. This block in communication also precludes the activation of the ATP hydrolysis rate that is normally seen when kinesin binds to the microtubule. According to our measurements, this mutant will accumulate microtubule-kinesin complex with ATP bound to one site and ADP bound to the other (between steps 1 and 2 in Fig. 2). Apparently, conformational changes induced by microtubule binding are needed for the activation of the ATP hydrolysis step and may be related to the microtubule-dependent stimulation of ADP release. This result does not override our previous conclusion the ATP binding and not hydrolysis was necessary and sufficient to stimulate ADP release from the second head (2). Rather it suggests that perhaps the release of ADP from the second head is coupled to changes in conformation of the first head needed to stimulate ATP hydrolysis. Thus, it may be that ADP release necessarily precedes ATP hydrolysis as shown in Fig. 2, and this may represent an additional site for coupling to keep the reactions on the two heads out of phase.

Two kinesin switch II mutants in which Glu237, thought to participate in an electrostatic interaction with switch I Arg204, were replaced and examined using steady- and transient-state kinetic methods. Substitutions of Glu237 with either alanine or aspartate disrupted microtubule-dependent ATP hydrolysis by at least an order of magnitude, whereas substitutions at Glu200 were found to be less disruptive to ATPase activity (21–24). Analysis of pre-steady-state ATP hydrolysis critical in stabilizing a water molecule required in the catalysis of the ATPase reaction (21–24). This block in communication is then coupled to subsequent isomerization reactions to stimulate ADP release from the neighboring site. Further detailed structural and kinetic studies on rat kinesin will allow further definition of the reactions underlying the coupling of alternating states to force production.

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