Feedback of the Kinesin-1 Neck-linker Position on the Catalytic Site*

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Kinesin-1 (formerly conventional kinesin) proteins are molecular motors that move along microtubules in a stepwise fashion at the expense of ATP hydrolysis energy. A large body of evidence shows that these motors are dimeric and generate motility by the alternating action of two identical motor domains (1–6). A crucial structural intermediate is a Kinesin-1 molecule that is strongly attached to the filament by one nucleotide-free head, whereas the other one remains in a weakly microtubule-binding ADP state. Upon ATP binding, a very rapid structural change of the neck-linker of the microtubule-bound motor head occurs that allows the partner head to lose its ADP and to bind tightly to the microtubule (7). The neck-linker, a short stretch of ~12 amino acid residues that follows immediately C-terminal of the catalytic core motor domain, is thought to exist in at least two different conformations, one that is docked or zippered to the motor core, and a flexible undocked structure (8, 9).

The docked neck-linker is visible in some crystal structures (10, 11), and its path along microtubule-bound kinesin has been deduced from cryo-EM (8, 12). According to these data, the neck-linker zippers along a groove in the core motor domain between helix 6 on one end, and loop 10 on the other end where it is held by a meshwork of hydrogen bonds. The nature of the undocked conformation is not clear and might differ in truncated, monomeric constructs (that have been used for many of the structural investigations) and dimeric ones.

The conformational change of the neck-linker has been used to explain the mechanism of Kinesin-1 motility. It is thought that neck-linker docking allows or causes the second head to bind to a forward microtubule binding site. Interestingly, it is unlikely that the docking process of the neck-linker generates the force exerted by kinesin. The free energy of neck-linker docking is surprisingly low (∆G ~ −1 kJ/mol; Ref. 9), and hardly accounts for the force that can be generated by a single kinesin molecule (5–6 pN, Ref. 13). Estimated from crystal structures, the length of the neck-linker is at least 4 nm. Accordingly, ∆G will be ~12 kJ/mol, which is roughly 10-fold larger than the measured free enthalpy change of neck-linker docking.

Finally, the neck-linker has been shown to play other roles than providing energy or directionality for motility. Most simplistic, it provides the spatial freedom to bridge the 8-nm gap between adjacent microtubule binding sites. Motility assays with neck-linker mutants that were arrested in the docked state by disulfide bonds failed to step, supporting the structural importance of the neck-linker region (14). However, the reality is more complex because from the crystal structure and the known geometry of the kinesin-microtubule complex a strain is predicted to arise between the two heads when they are in the intermediate state where both heads bind simultaneously. This strain has been suspected to change the kinetic properties of the motor domains and facilitates dissociation of the rear head, and/or slows down the ATP binding of the leading head (15–18). The models assume that the leading, nucleotide-free head of the conventional kinesin motor possesses a flexible neck-linker that exerts a strain on its rear partner. The forward strain on the trailing head has been proposed to accelerate dissociation from the microtubule by a factor of (~2 (15–18). In addition, the rearward strain on the leading head seems to inhibit ATP binding, which keeps the head in a strong microtubule binding state and prevents premature release (16). According to these models, the neck-linker thus plays a central role in the generation of motility by signaling the binding state of the leading head to the lagging one.

The present study tests whether neck-linker immobilization merely prevents motility by restricting the diffusional freedom of the leading head, or whether there are other, less direct kinetic effects that might be related to strain transmission. Our results on cross-linked neck-linker mutants show that the latter is the case because kinesin monomers (that function without any communication between motor heads) show dramatic effects when their neck-linker is kept in the docked state. We investigate the structural requirements for this feedback in mutants with an altered interface between neck-linker/motor core. Our results indicate that the kinesin motor core contains important sensor residues for the position of the neck-linker that directly affect the microtubule affinity, and thus also the kinetics of the core motor domain.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Kinesin was expressed and purified as described (19, 20). Briefly, Neurospora crassa kinesin (Nckin) was bacterially expressed using a T7 promoter system, isolated

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from *Escherichia coli* cells and purified on a HitTrap SP-Sepharose column (Amersham Biosciences) in 20 mM sodium phosphate buffer, pH 7.4 and 5 mM MgCl₂. The protein was eluted with increasing NaCl concentrations. Human kinesin mutants were purified according to published procedures (14). DTNB was present during the cell rupture but omitted during the ion exchange chromatography. Tubulin was prepared and polymerized as described (21, 22).

**NcKin Protein Backgrounds**—As protein background cysteine-light NcKin constructs were used that did not contain maleimide-reactive cysteine residues, as confirmed by dye labeling experiments. Two variants of cysteine-light backgrounds were used: one retaining two native cysteine residues (Cys³⁸ and Cys⁵⁹; mutation: C307A), one lacking all endogenous cysteine residues (C38V/C59T/C307A; Cys-free).

Furthermore, truncated NcKin backgrounds of different lengths were used. The C-terminal deletion construct NcKin-343 (NcKin residues 1–343; Ref. 19) was used to study the behavior of monomeric constructs, NcKin-436hKT (NcKin residues 1–436 and human kinesin residues 432–546; Ref. 23) that of dimeric kinesins. In this chimera, part of the human kinesin stalk was appended to be able to bind NcKin mutants to glass coverslips. The addition of the human tail portion only affects the behavior of the motor in few, very specific mutant contexts (23). As a control, the AE mutant comprising residues 1–436 of NcKin was tested and did not show a significant kinetic difference in the microtubule-dependent ATPase assay between variants lacking or containing the partial human stalk. Therefore, all other mutants were only tested in the chimeric background containing the human stalk.

**Introduction of Disulfide Bridges**—To introduce cross-links between the kinesin motor core and neck-linker, pairs of amino acid residues were selected that, according to known crystal structures, are in close proximity to each other to allow the formation of disulfide bonds (Fig. 1; Refs. 10 and 24). The selected wild-type residues were changed to cysteines in Kinesin-1 and the S.E. of this measurement was used to estimate a reliable interval of measured thiols per protein. Because the DTNB-oxidized protein preparations could not be measured directly in the thiol quantification assay, an additional SP-Sepharose chromatography step was used to remove excess TNB. Kinesin-containing elution fractions were pooled, quantified by a Bradford assay, and subject to the DTNB thiol assay.

For mass spectrometry, the protein was carbamidomethylated. To this end, the protein was incubated with 50 mM iodoacetamide, which turned out to lead to the best yield of modified protein in a reasonable time, and an acceptable degree of side products. The reaction was stopped with 100 mM DTT, and the sample was purified over a C18 column (OMIX Tips C18MB, Varian) according to the manufacturer's instructions. Mass spectrometry was performed as described (26).

**Gliding and Kinetic Assays**—Standard motility assays were performed by applying 1.25 μg of kinesin (NcKin-436hKT) to a coverslip that was kept for 1–2 min in a wet chamber to allow the human kinesin stalk to bind to the glass surface. Casein buffer was added to a final volume of 5 μl. Then 1 μl of BRB80 (80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) with 100 μM KCl, 1 μl of 100 mM MgCl₂, 1 μl of 100 μM ATP, and 1 μl of 1 μM microtubules were added. The coverslip was put onto a glass slide and sealed. Motility was monitored in a Zeiss Axioshot using video-enhanced phase-contrast microscopy.

Some constructs were also monitored in a TIRF microscope (Olympus), where tetramethylrhodamine- or Atto488-labeled microtubules were tracked. This assay allowed much higher microtubule concentrations, facilitating characterization of mutants with low microtubule affinities. Motor densities were not strictly controlled in these assays. Fluorescence was excited using linearly polarized 532 nm or 488 nm laser light, a ×100 Zeiss objective and an Electron Multiplier CCD Camera (Hamamatsu C9100-02).

The ATPase activity was routinely measured in dependence on the microtubule concentration. The steady state ATP turnover was characterized in a coupled enzymatic assay in 12A25 buffer (12 mM Acet/ KOH, pH 6.8, 5 mM MgCl₂, 0.5 mM EGTA, 25 mM potassium acetate; Refs. 19 and 27) in the presence of saturating ATP concentrations (2 mM ATP), and titrated with variable amounts of microtubules until a reliable Michaelis-Menten curve was established (typically, R > 0.95). The maximal turnover number, K₉₀₅₆₉₉, and the half-maximal activation constant, K₉₅₉₆₉₉₉₉, as well as their experimental errors, were deduced from a hyperbolic curve fit using Kaleidagraph software (Synergy Software, Reading, PA). The Michaelis-Menten constants for ATP, K₉₉₅₆₉₉, were measured at saturating microtubule concentrations (5 or 9 μM for dimeric constructs, which is more than 10-fold higher than K₉₅₆₉₉₉₉₉ higher microtubule concentrations were impractical to use).

The basal ATP turnover was assayed in 12A25 by quantification of radioactive inorganic phosphate released from hydrolyzed [γ-³²P]ATP over time (19). Phosphate was separated from nucleotide and protein using charcoal (28). The linear parts of these curves were used to calculate the basal ATP turnover if they contained at least 4 data points in the linear part, and if the double amount of kinesin in the assay led to a curve slope double as steep.

The microtubule-induced mant-ADP release was measured by mixing mant-ADP charged kinesin and microtubules in the presence of unlabeled ATP, either manually in a spectrophotometer (Amino-Bowan AB2) or in a stopped-flow instrument (Biologics SF-3) under conditions published previously (29).

The microtubule affinity of kinesin was determined using a fixed amount of kinesin (300–500 nM), and increasing amounts of microtubules (0–10 to 50 μM, depending on the mutant and condition used (30)). The tubulin dimer concentration was used to analyze the assays. Kinesin and microtubules were mixed in 12A25 buffer, and the micro-
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FIGURE 1. Positions of cross-linked residues in crystal structure models of Kinesin-1. The figure shows crystallographic models of the motor domains of rat kinesin (left, PDB access code 2KIN) and N. crassa kinesin (right, 1GOJ), which was used for most part of the study. The neck-linker is shaded dark gray, residues mutated into cysteine to form disulfides are red, residues in the motor core that were replaced by glycine to test kinetic relaxation of the AE cross-link are blue. The homologous residues in 2KIN are indicated and colored according to the same scheme. The models were generated in SwissPDB Viewer.

For unknown reasons, the supernatant contained variable amounts of tubulin, ranging from estimated 1–10%. If this was unpolymerized tubulin, the actual microtubule concentration would be overestimated ≤ 10%; if it contained small microtubule fragments that kinesin can still bind to the bound fraction would be underestimated by ≤ 10%.

Statistical Analysis of Experimental Results—To decide whether an observed effect was significant, tables of gliding velocity and ATPase behavior of each single protein assay and preparation were considered (supplemental table; analysis software: GraphPad Prism 4.0, GraphPad Software Inc., San Diego, CA). In cases where the number of independent protein preparations was 3 or more, a paired Student’s t test could be used to test the null hypothesis that (otherwise identical) DTT- and DTNB-treated protein samples behaved identically. For this study, 32 independent dimer protein preparations were analyzed in gliding and ATPase assays. 29 monomer preparations were characterized in ATPase assays. Because the fungal Kinesin-1 NcKin is much faster than conventional kinesins from animals in gliding and enzymatic assays, the effects are clearer in this background.

RESULTS

Mutant Design and Proof of Expected Disulfide Bonds—The Kinesin-1 neck-linker plays an important mechanical role in the generation of movement, and a permanently docked neck-linker prevents microtubule motility (14). In the Tomishige/Vale study, the neck-linker was kept in a docked state by inducing disulfide bonds between neck-linker and motor core. It was suggested that the cross-linked neck-linker of the microtubule-bound head restricts the mobility of the second, unbound kinesin head in a way that does not allow stepping. Although often assumed, it is unknown, however, whether there are other kinetic effects of neck-linker immobilization. We therefore looked for possible kinetic effects of neck-linker docking, and generated cysteine-light NcKin kinesin constructs in which we introduced pairs of cross-linkable cysteine residues at the interface between neck-linker and motor core (Fig. 1), and tested the activity in multiple motor gliding assays and steady state ATPase assays.

To immobilize the neck-linker, three different positions were chosen (S3C-A334C, N. crassa NcKin numbering; brief: SA–cross-link, R300C-A334C, RA–cross-link, A226C-E339C, AE–cross-link). Judged from the distances in crystal structures (Protein Data Bank numbers: 1GOJ, 2KIN, 1MKJ), these are the only pairs of residues that can form disulfide bonds without major structural distortions.

To exclude artifacts of nonspecific cross-linking, all three endogenous NcKin cysteine residues were exchanged. In the first attempt the cysteine residues were mutated to serine or alanine. It turned out, however, that proteins containing C38S and C59S were not expressed by E. coli, whereas the C307A exchange did not affect expression and kinetic behavior. For most parts of the study we therefore used a background that still contained Cys307 and Cys59, but lacked Cys38 (NcKinC307A). To test whether the intended disulfides actually formed, we applied the following strategy: We first visualized the cross-link on a non-reducing SDS gel (Fig. 2), then used DTNB-based thiol quantification to show that all expected cysteines are present in untreated proteins, then showed that two cysteines of the SA and AE mutant become inaccessible to DTNB after oxidation, and finally identified the residues that were only accessible under reducing conditions by mass spectrometry.

Oxidized NcKin AE and SA mutants, but not the wild-type, showed an increased mobility in SDS-PAGE (Fig. 2). However, the oxidized samples still contained a variable portion with mobility comparable to wild-type or reduced proteins. We therefore determined the thiol content in a colorimetric assay. Table 1 shows that in most constructs all cysteine residues are accessible to DTNB. The dimeric AE mutant is an exception, and shows considerable differences between both tested preparations, indicating partial oxidation of the protein. To test whether DTNB treatment led to proteins with two inaccessible cysteines, control preparations of dimeric AE and SA mutant were made and assayed immediately after the purification, as well as after DTNB treatment and subsequent removal of DTNB by a second chromatography step. To compensate the loss of material during this procedure and to be able to detect even low thiol concentrations, complete preparations dedicated to this test were used. The assay showed that the oxidized AE mutant...
Functional Analysis of Cross-linked Proteins—For functional assays, the proteins were routinely treated with 1 mM DTT to break possible disulfide bonds (reduced state), 0.2 mM DTNB to induce their formation (oxidized), or re-reduced with 5 mM DTT after DTNB oxidation (reduced). A test series confirmed that the oxidative effect of DTNB was completed after 5 min, whereas some constructs were only re-activated to a reasonable degree after 4 h. The DTT-reduced monomeric human construct hK339-K222C/E334C even showed higher ATPase activity after 72 h than 22 h. As controls, important constructs were also assayed untreated (i.e., exposed to air-saturated buffer). To test whether a thiol modification of any endogenous or introduced cysteine residue had an effect by itself, some constructs were incubated with N-ethylmaleimide, which did not have an effect for any of the tested constructs.

Motility Assays—As a start, the effect of disulfide bonds on the motor activity of NcKin-hKT mutants was measured. By contrast to the wild-type control and all single mutants that were unaffected by DTNB, all cross-linked kinesin mutants slowed down to velocities between 3 and 15% in multiple motor gliding assays (Table 2). The most drastic effect was observed in the SA mutant that only showed 3% residual gliding velocity. The calculated residual velocity of 12% (±0.26 μm/s) of the AE cross-link mutant is an upper limit because huge variations in the behavior of individual microtubules were observed (supplemental movie). In general, moving microtubules were extremely rare, and those that were displaced usually stalled after a very brief period of time (<1–2 s). As described further below, this behavior is most likely caused by the presence of a mixed population of cross-linked, inactive motors, with a small contamination of uncross-linked, and therefore wild-type-like proteins.

ATPase Assays on Kinesin Dimers—Cross-linked, dimeric motors were also characterized in steady state microtubule-activated ATPase assays (Table 3 and Fig. 3). Here, large differences were observed. The AE cross-link mutants (in the C307A and Cys-free backgrounds) that fixed the neck-linker over its entire length were strongly inhibited and showed only 14% residual ATPase rate after oxidation (4.6 s⁻¹/29.3 s⁻¹). The ANOVA test comparing the $k_{cat}$ of the oxidized NcKin-hKT AE mutant with that of the reduced mutants indicated that oxidation caused a 14-fold $k_{cat}$ difference with a significance level of $p < 0.05$. The $k_{cat}$ of the analogous human kinesin mutant was inhibited 4-fold by DTNB, in agreement with the measurement on the NcKin mutants but in contrast to previous observations (~2-fold inhibition, (32)). The $k_{cat}$ of the RA mutant was ~3.9-fold slower under oxidizing conditions (13.0 s⁻¹/50.5 s⁻¹) and significant in the ANOVA. The response of the SA cross-link $k_{cat}$ was statistically insignificant and much smaller than that of the RA mutant (on average 96.6 s⁻¹/DTNB)/90.4 s⁻¹ (DTT)). This is especially remarkable because both mutants cross-link the same neck-linker residue A334C. All cross-links were fully reversible by 5 mM DTT. The ANOVA and the Student’s t test did not allow rejecting the null hypothesis that the cysteine-light parent construct or the control constructs with only one introduced cysteine residue show identical $k_{cat}$.

**TABLE 1**

Thiol quantification

Quantification of thiol groups by DTNB. Two independent, untreated preparations of each kinesin mutant were incubated with DTNB to determine the amount of thiol groups by the extinction $ε_{412}$ of the TNB reaction product (for details of the protocol, see “Experimental Procedures”). The calculated thiol concentration was compared to the protein concentration to give the number of SH groups per protein. As the largest error arises from the accuracy of the protein concentration, an interval was derived from the S.E. of the protein concentration assay (column 4).

| Construct | Cysteine residues | Measured SH/protein | Confidence interval | Comment |
|-----------|------------------|---------------------|--------------------|---------|
| Monomeric |                  |                     |                    |         |
| NK343C307A, AE | 2            | 1.70                | 1.61–1.78          |         |
| NK343C307A, AE | 4            | 3.62                | 3.28–3.90          |         |
| NK343C307A, SA | 4            | 4.15                | 3.85–4.58          |         |
| NK343C307A, wt | 0            | 0.08                | 0.06–0.10          | TNB extinction close to the detection limit |
| Dimeric   |                  |                     |                    |         |
| NKhTailC307A, AE | 2            | 0.85                | 0.27–1.50          | Preparation 1: 1.30 SH/protein preparation 2: 0.28 SH/protein |
| NKhTailC307A, SA | 4            | 3.98                | 3.53–4.46          |         |
| NKhTailC307A, RA | 2            | 1.51                | 1.20–1.87          |         |
| NKhTailC307A, RA | 0            | 0.14                | 0.12–0.15          |         |
| NKhTailC307A, E334C | 3            | 2.87                | 2.55–3.21          |         |
| NKhTailC307A, A226C | 3            | 3.35                | 3.22–3.47          |         |
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### TABLE 2
Effect of oxidation on the gliding velocities of NcKin cysteine mutants

The table summarizes the gliding velocities of DTT- and DTNB-treated NcKin mutants and oxidized proteins after rescue with excess DTT. The values give average microtubule gliding velocities ± S.E. in µm/s (usually averages of 20–25 microtubules; for some weak microtubule-binding preparations 7–15), and the number of independent preparations tested (n). The original data of each single gliding assay were analyzed by one-way ANOVA to evaluate the significance of the velocity difference under DTNB and DTT conditions. p values were calculated using Tukey’s post-analysis method, as implemented in GraphPad Prism 4.2 and are indicated alongside the gliding velocities under DTNB conditions.

| Constructs          | Gliding velocities | Significant difference? | Rescue |
|---------------------|--------------------|--------------------------|--------|
|                      | DTT (µm/s)         | DTNB (µm/s)              |        |
|                      | n = 2              | n = 2                    |        |
| NcKin-436hKT         | 2.53 ± 0.10        | 2.41 ± 0.06              | No     |
|                      | n = 3              |                          |        |
| NcKin-436hKT         | 2.13 ± 0.21        | ≤ 0.26 ± 0.09            | Yes    |
| A226C/E339C          | n = 3              |                          |        |
| NcKin-436hKT         | 2.53 ± 0.42        | 2.61 ± 0.05              | No     |
| A226C                | n = 3              |                          |        |
| NcKin-436hKT         | 2.57 ± 0.05        | 2.19 ± 0.08              | No     |
| E339C                | n = 3              |                          |        |
| NcKin-436hKT         | 2.32 ± 0.18        | 0.07 ± 0.01              | Yes    |
| S3C/A334C            | n = 2              |                          |        |
| NcKin-436hKT         | 2.77 ± 0.08        | 2.06 ± 0.15              | No     |
| A334C                | n = 3              |                          |        |
| NcKin-436hKT         | 2.46 ± 0.14        | 1.59 ± 0.26              | No     |
| R300C/A334C          | n = 2              |                          |        |
| R300C                | n = 3              |                          |        |
| NcKin-436hKT         | 1.54 ± 0.15        | 0.18 ± 0.01              | Yes    |
| R300C                | n = 2              |                          |        |
| NcKin-436hKT         | 1.77 ± 0.24        | 1.88 ± 0.04              | No     |
| R300C                | n = 3              |                          |        |
| NcKin-436hKT         | 0.64 ± 0.16        | 0                          | ND     |
| NcKin-436hKT         | n = 2              |                          |        |
| Y80G                 | 0.97 ± 0.28        | 0.23 ± 0.03              | Yes    |

* See supplemental movie.
* ND, not determined.
* ND, not determined.

### TABLE 3
Microtubule-dependent ATPase activity of cross-linked and uncross-linked dimeric NcKin-hKT mutants

The table compares microtubule-activated ATPase rates, k_cat ± S.E. in s⁻¹ and the number of independent preparations tested, n, of the kinesin mutants used in this study. A full list of all individual measurements is supplied in the supplemental table. Although the average half-maximal activation constants for microtubules, K_0.5,MT, were not recognizably different, the paired comparison in each assay, tested on the same day with the same batch of microtubules, revealed significant differences of K_0.5,MT for AE-containing mutants (see text).

| Constructs          | Steady state Mt-ATPase k_cat (s⁻¹) | Average change of K_0.5,MT and k_cat |
|---------------------|-----------------------------------|-------------------------------------|
|                      | DTT                               | DTNB                                | K_0.5,MT(DTNB)/K_0.5,MT(DTT) | k_cat(DTNB)/k_cat(DTT) | n |
|                      | n = 2                             | n = 2                               |                         |                        |   |
| NcKin-436hKT         | 61.7 ± 14.2                       | 60.4 ± 12.6                         | 0.12 ± 0.06             | 0.84 ± 0.02            | 3  |
| Wild type            | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 29.3 ± 3.3                        | 4.6 ± 0.5                           | 3.62 ± 2.22             | 0.22 ± 0.18            | 3  |
| A226C/E339C          | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 53.0 ± 3.9                        | 52.3 ± 11.0                         | 1.07 ± 0.43             | 1.37 ± 0.49            | 3  |
| E339C                | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 67.7 ± 14.4                       | 56.2 ± 7.8                          | 1.58 ± 0.36             | 0.59 ± 0.10            | 3  |
| S3C/A334C            | n = 2                             | n = 2                               |                          |                        |   |
| NcKin-436hKT         | 90.4 ± 21.2                       | 96.6 ± 10.9                         | 0.55 ± 0.15             | 2.10 ± 0.31            | 2  |
| S3C                  | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 84.0 ± 7.7                        | 65.6 ± 17.7                         | 1.43 ± 0.13             | 0.61 ± 0.10            | 3  |
| A334C                | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 50.5 ± 8.3                        | 13.0 ± 0.1                          | 5.40 ± 1.59             | 0.04 ± 0.00            | 2  |
| R300C/A334C          | n = 2                             | n = 2                               |                          |                        |   |
| NcKin-436hKT         | 38.2 ± 10.7                       | 32.0 ± 8.6                          | 0.58 ± 0.12             | 1.65 ± 0.43            | 3  |
| R300C                | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 23.0 ± 4.0                        | 14.6 ± 4.3                          | 0.60 ± 0.13             | 1.11 ± 0.22            | 3  |
| A226C/E339C-Y80G     | n = 3                             | n = 3                               |                          |                        |   |
| NcKin-436hKT         | 69.1 ± 12.6                       | 71.2 ± 13.1                         | 1.17 ± 0.09             | 1.08 ± 0.45            | 2  |
| Y80G                 | n = 2                             | n = 2                               |                          |                        |   |
| hKin560-GFP_ATP      | 58.8 ± 0.9                        | 14.7 ± 4.8                          | 5.47 ± 0.95             | 0.06 ± 0.01            | 2  |

* ND, not determined.

values under DTT and DTNB conditions. This agrees with gliding assays where no detectable DTNB-induced difference occurred.

The Michaelis-Menten constants for ATP, K_M,ATP were not affected by oxidation (83 ± 10 µM), but the half-maximal activation constants for microtubules, K_0.5,MT showed a significant response to DTNB in AE-containing mutants. The relative K_0.5,MT values (averages ± S.E.) shown in Tables 3 and 4 suggest that the K_0.5,MT is not significantly affected by formation of the neck-linker cross-links. This is caused by
the large variability in the assay. From day to day, we observed variations of $K_{0.5,MT}$ in the range of a factor of 3. Although changed microtubule polymerization and storage conditions did not change this general effect, we suspect that this variability is caused by an unknown parameter of our microtubule preparation. Therefore, $K_{0.5,MT}$ values were only comparable when they were obtained on the same batch of microtubules and on the same day. Therefore, to test whether the $K_{0.5,MT}$ was affected by DTNB treatment, a pair-wise comparison of $K_{0.5,MT}$ values of DTT- and DTNB-treated samples was analyzed (supplemental table). Only assays done on reduced and oxidized samples in parallel were included. With this criterion, all AE-containing mutants (except for the AE-Y80G mutant, discussed below) showed a significant or highly significant increase of $K_{0.5,MT}$ by a factor of 2–5 upon oxidation. The $k_{cat}$ (defined as the ratio $k_{cat}/K_{0.5,MT}$) changed even more drastically because AE mutants show a slower $k_{cat}$ and a higher $K_{0.5,MT}$. This indicates a lower microtubule affinity and/or a reduced processivity (1).

ATPase Defect in Monomers—One possible explanation why the ATP turnover rates of the AE and RA cross-link mutants are disturbed is that the second, unbound kinesin head is unable to bind to a new microtubule site, and therefore consumes ATP more slowly (32). Although the unaffected ATPase activity of the SA cross-link mutant argues against this, the direct way to address this issue is to measure monomer ATPase rates (Table 4 and supplemental Fig. S2). We therefore tested the most extreme mutant, the AE cross-link, and measured its steady state microtubule-activated ATPase rate in the monomeric NcKin-343 background (19). The $k_{cat}$ value was $183 \text{s}^{-1}$ under reducing conditions, and decreased to $6 \text{s}^{-1}$ upon disulfide formation. The difference was highly significant ($p < 0.001$) in the ANOVA. The activity could be rescued to more than 50% by an excess of DTT (Table 4). The corresponding human mutant was inhibited ~20-fold. The decrease of the cross-linked NK343-AE mutant to roughly 3% was even more dramatic than in the dimer, and shows that the docked neck-linker feeds back directly to the catalytic motor domain, and not indirectly via the second motor head.

Together, the comparison of gliding and enzymatic activities of the cross-link mutants revealed an unexpected coupling between catalysis and motility. Whereas the immobilization of the neck-linker at the motor core (oxidized AE and RA mutants) affected both the gliding and the ATPase activity, the cross-link with the N-terminal Ser3-position (oxidized SA mutant) retained full enzymatic activity although it led to almost complete destruction of motility. This observation suggests the existence of a specific transmission pathway that signals the neck-linker position to the catalytic site. To further elucidate this pathway, we tried to identify residues in the crystal structure that might be involved.

Role of Tyr$^{80}$ in Coupling the Neck-linker Docking Response—The crystal structures of NcKin, rat or human Kinesin-1 motor domains (1GOJ, 2KIN, 1MKI) show that the neck-linker is docked to the motor core between helix H6 and loop L10 (Fig. 1). To investigate whether any amino acids along the length of the neck-linker are involved in coupling neck-linker position and ATPase activity, we removed the side chains of three residues by mutating them into glycine. The preferred alanine substitution Y80A was not expressed in Escherichia coli. Specifically, we mutated Asn298, Tyr80, and Asn81, which are the only residues in available crystal structures that extend their side chains toward the neck-
Kinesin Neck-linker Function

TABLE 4
Microtubule-dependent ATPase activity of cross-linked and uncross-linked monomeric NcKin mutants

The table compares microtubule-activated ATPase rates and half-maximal activation constants of monomeric cysteine mutants and shows average $k_{cat}$ values ± S.E. in s⁻¹, the number of independent preparations tested, $n$, and the relative $K_{cat,MT}$ of DTNB- and DTT-treated samples, and the number of independent preparations tested.

| Construct | Steady state Mt-ATPase $k_{cat}$ | Change of $K_{cat,MT}$ and $k_{cat}$ | $n$ |
|-----------|-------------------------------|---------------------------------|-----|
|           | DTT                           | DTNB                           | Rescue | $K_{cat,MT}$(DTNB)/$K_{cat,MT}$(DTT) | $k_{cat}$(DTNB)/$k_{cat}$(DTT) |
| NcKin-343 | 302.5 ± 13.4                   | 260.5 ± 13.7                   | 241.6 ± 7.4 | 0.92 ± 0.09 | 0.95 ± 0.08 | 2 |
| Wild type | $n$ = 2                       | $n$ = 2                        | $n$ = 2 |
| NcKin-343 | 183.3 ± 23.9                   | 5.5 ± 0.7                      | 123.1 ± 20.9 | 1.58 ± 0.39 | 0.00 ± 0.00 | 5 |
| A226C/E339C | 138.4 ± 9.4                   | 53.7 ± 10.0                    | 80.2 ± 23.2 | 3.78 ± 0.68 | 0.14 ± 0.005 | 6 |
| A226C/E339C-Y80G | $n$ = 6                   | $n$ = 6                        | $n$ = 5 |
| NcKin-343 | 200.7 ± 7.6                    | 193.2 ± 18.0                   | 199.9 ± 19.3 | 1.22 ± 0.02 | 0.79 ± 0.02 | 2 |
| Y80G | $n$ = 2                       | $n$ = 2                        | $n$ = 2 |
| NcKin-343 | 86.0 ± 10.9                    | 9.6 ± 0.5                      | 53.4 ± 13.4 | 3.33 ± 0.63 | 0.00 ± 0.00 | 3 |
| A226C/E339C-N81G | $n$ = 3                   | $n$ = 3                        | $n$ = 3 |
| NcKin-343 | 180.0 ± 13.7                   | 24.5 ± 6.7                     | 91.3 ± 9.8 | 4.39 ± 1.09 | 0.00 ± 0.02 | 2 |
| A226C/E339C-N298G | $n$ = 2                   | $n$ = 2                        | $n$ = 2 |
| hKin339his | 73.8 ± 4.0                     | 3.6 ± 0.6                      | 16.7 ± 3.9 | 5.58 ± 3.84 | 0.46 ± 0.34 | 3 |
| K226C/E334C | $n$ = 3                     | $n$ = 3                        | $n$ = 2 |

TABLE 5
Significance of DTNB-induced changes of $k_{cat}$

The table gives the results of the one-way ANOVA of the relative inhibitory effect of DTNB on groups of mutants. The ratio of $k_{cat}$ values was calculated pairwise from DTNB-treated and DTT-treated protein preparations assayed in parallel (supplemental tables). The significance was calculated in GraphPad Prism 4.2. Thus, a mean difference of 0.0 indicates identical $k_{cat}$ values of oxidized and reduced mutant construct, a mean difference of 1.0 means complete inactivation of the oxidized enzyme.

| Tukey’s multiple comparison test | Mean difference | $p$ value | 95% confidence interval of difference |
|---------------------------------|-----------------|-----------|-------------------------------------|
| Wild type vs AE mutants         | 0.8600          | $p < 0.001$ | 0.6389 to 1.081                      |
| Wild type vs SA mutants         | $-0.09738$      | $p < 0.05$  | $-0.3805$ to 0.1857                  |
| Wild type vs RA mutants         | 0.7017          | $p < 0.001$ | 0.3773 to 1.026                      |
| Wild type vs Y80G mutants       | $-0.07505$      | $p < 0.05$  | $-0.3171$ to 0.2030                  |
| Wild type vs AE-Y80G mutants    | 0.4826          | $p < 0.001$ | 0.2664 to 0.6988                     |
| Wild type vs AE-N81G mutants    | 0.8045          | $p < 0.001$ | 0.4783 to 1.131                      |
| Wild type vs AE-N298G mutants   | 0.8244          | $p < 0.001$ | 0.5396 to 1.109                      |
| AE mutants vs AE-Y80G mutants   | $-0.3774$       | $p < 0.001$ | $-0.5657$ to $-0.1890$               |
| AE mutants vs AE-N81G mutants   | $-0.05550$      | $p < 0.05$  | $-0.3637$ to 0.2527                  |
| AE mutants vs AE-N298G mutants  | $-0.03559$      | $p < 0.001$ | $-0.2995$ to 0.2284                  |
| AE-Y80G mutants vs AE-N81G mutants | 0.3219       | $p < 0.05$  | 0.01708 to 0.6266                    |
| AE-Y80G mutants vs AE-N298G mutants | 0.3418      | $p < 0.01$  | 0.08185 to 0.6017                    |
| Y80G mutants vs AE-Y80G mutants | 0.5396         | $p < 0.001$ | 0.3067 to 0.7726                     |

linker (Fig. 1). We introduced these mutations into the AE cross-link protein background, because we reasoned that if any of these residues would be important for coupling of neck-linker position and ATPase activity, the ATPase rate of the cross-linked triple mutant should be faster than the slow ATPase rate of the cross-linked AE mutant (relaxed inhibition). To exclude artifacts because of alternating site catalysis, we used the monomeric protein background for these assays.

Steady state ATPase assays on reduced and oxidized AE-Y80G, AE-N81G, and AE-N298G mutants in fact showed the predicted relaxed inhibition (Table 3 and 4, supplemental Fig. S2). Whereas the oxidized AE parent mutant showed only 3% residual ATPase rate in the monomer, both N → G triple mutants were ~3–4-fold faster, the Y80G triple mutant even more than 10-fold (38% residual activity in the monomer, 63% in the dimer). The result of the ANOVA (Table 5) showed significant differences, and Tukey’s post-analysis test indicated that all the three triple mutants were highly significantly more affected by DTNB than the wild type, that the AE-Y80G mutants were the least affected (mean difference from wild type –50% versus ~80% for AE-N81G and AE-N298G). The ANOVA also showed that the AE-Y80G mutants (but none of the AE-N mutants; not shown) were highly significantly less affected than the AE double mutant.

The most straightforward explanation is that the absence of the side chains uncoupled ATPase activity and docked neck-linker position. To further understand the AE-Y80G mutant we looked at the activity in the dimeric background (Tables 2 and 3). As in the monomeric background, the ATPase rate was faster than in the AE cross-link alone (63% versus 16% residual ATPase rate), whereas the gliding activity was completely abolished. This again emphasizes the role of Tyr⁸⁰ as a mediator of neck-linker position. One could argue that the relaxation of the ATPase inhibition originates in a lower extent of cross-linking, leading to more active uncross-linked motors in the assay. The lack of gliding activity of the dimeric AE-Y80G mutant argues against this. If there were a significant fraction of uncross-linked motor present, one would expect to see at least very slow gliding in the multiple motor assay (see below and Fig. 4). Therefore, the AE cross-link seems to be induced in our oxidation procedure, also in the absence of the Tyr⁸⁰ side chain. Together, these observations argue that Tyr⁸⁰ is the major transmitter of the neck-linker position.

Noteworthy, the (uncross-linkable) Y80G single mutant was equally active as wild type NcKin in the ATPase assay but 2.5-fold slower in multiple motor gliding assays under reducing conditions (Tables 2, 3, and 4). Its gliding velocity of ~1.0 μm/s was slower than that of the wild type, in agreement with the slower velocity of the uncross-linked triple AE-Y80G mutant (0.64 μm/s versus 2.3–2.5 μm/s). This emphasizes that Tyr⁸⁰ is located at a sensitive position and may be required for the coupling of enzymatic and mechanical processes. For our interpretations this mutant defect is not relevant, though. In our cross-linking assays the neck-linker was kept in its docked state, which was achieved to the full degree because of sufficiently long oxidation exposure. Under this experimental condition where the docking kinetics does not influ-
en the observations, the function of Tyr⁸⁰ as a coupler became clearly visible.

**Mixed Population Motility Assays**—Which part of the catalytic cycle does the docked neck-linker influence? Gliding assays of the dimeric AE and SA mutants gave a first hint. Curiously, in these assays, the gliding activity of cross-linked mutants was not completely abolished. This could be caused by the fact that the cross-linked motors still show residual activity, or that oxidation produces a mixed population of cross-linked, fully inactive motors, and uncross-linked motors with wild type properties. To learn how a mixture of native and inactive motor behaved, we performed mixed gliding assays. In these assays, the crosslinkable AE and SA cross-link mutants were mixed with wild type motor (without reactive cysteine residues) and measured in multiple motor gliding assays. The results showed that adding inactive, cross-linked mutants to active wild type motors lead to a gradual decrease in gliding velocity. However, both cross-link mutants were very different in their efficiency to slow down the wild type motor population. Whereas a portion of ~50% of the cross-linked SA mutant led to 50% slower gliding, as few as 1% of wild type kinesin mixed with oxidized AE-mutant still drove half-maximal gliding velocity (Fig. 4). These observations prove that very few uncross-linked motors may have been responsible for the observed residual velocity in assays of the oxidized AE mutant. Moreover, they suggest that the AE mutant may have a reduced microtubule affinity.

**Microtubule Affinities**—To check whether the AE cross-link was arrested in a weak microtubule binding state, the affinities were determined in microtubule co-sedimentation assays (Fig. 5 and Table 6). The analysis showed that the oxidized, cross-linked AE mutant is compromised in its ability to bind microtubules. The microtubule affinity of the oxidized monomeric mutant decreased roughly 4-fold in the presence of ADP, the affinity of the dimer almost 2-fold (Table 6). These differences were significant in the ANOVA (p < 0.05), in contrast to the differences of the SA mutant. Noteworthy, the differences in the presence of AMP-PNP were not significant. These assays further substantiated that the cross-linked AE-mutant (but not the SA mutant) had a weaker affinity to microtubules than wild type.

In agreement, microtubule-induced mant-ADP release was highly disturbed in the dimeric AE-mutant. Under reducing conditions, mant-ADP release was induced by microtubules with a bimolecular binding rate constant of $k_{\text{cat}}$ of 3.5 μM⁻¹s⁻¹ for effective kinesin-microtubule binding under microtubule-limiting conditions, which is typical for wild-type kinesins (19). Under oxidizing conditions, no detectable signal amplitude was observed in the experiment. The initial emission signal where all active kinesin was saturated with mant-ADP was similar to the reduced sample, but only decreased at a rate comparable to the approximate rate of mant-ADP bleaching (0.0036 s⁻¹).

**DISCUSSION**

The neck-linker domain of Kinesin-1 motors plays a crucial role in the generation of motility. It is known to exist in at least two conformations, one that is captured in some crystal structures, comprises two short β-strands and is associated with the motor core, and another one that is less well described structurally, but is known to be flexible based on EPR studies (8, 9, 11, 24, 33). Kinesin is thought to switch between these two states during its chemo-mechanical cycle. According to previous studies, microtubule-bound, nucleotide-free kinesin has the flexible, unzipped neck-linker, and transits into the docked neck-linker state very rapidly upon binding of ATP (7). Neck-linker docking is thought to be the first step of the kinesin that induces stepping of the other, unbound head.

The importance to switch between docked and undocked positions was demonstrated in a study where disulfide bonds were introduced to immobilize the kinesin neck-linker (14). This manipulation led to the disruption of motility on the single molecule level, suggesting that the second kinesin head was unable to step forwards, and the dimer was arrested in a microtubule-bound state. This study likely is not to be a complete inhibition of kinetic activity. This observation is not due to the use of Neurospora kinesin because monomeric and dimeric human kinesin constructs based on (14) were fully inactivated upon cross-linking in our hands. The previous study found approximately half the ATPase rate in cross-linked dimers, and speculated that the cross-linked intermediate might prevent the second head from binding to the next microtubule site. We suspect that the protein under non-cross-linking conditions was strongly oxidized, as indicated by the reported very low $k_{\text{cat}}$ of 13.2 s⁻¹ (versus 58.8 s⁻¹ under our conditions).

Surprisingly, only cross-linking the neck-linker to a groove between helix 6 and loop 10 of the kinesin core motor domain (RA and AE mutants) inhibited the ATPase activity. Whereas cross-linking of A334C in the RA mutant slowed down the catalytic activity to a quarter, attaching the same residue to S3C did not show any significant change. This may be caused by a considerable degree of flexibility of the N-terminal residues that allows the transition to an undocked neck-linker state permitting ATP turnover but not stepping.

Remarkably, the kinetic effect of the AE cross-link occurred also in a monomeric protein background, excluding that the second head is involved and suggesting a direct feedback of neck-linker docking to the catalytic site. It was also observed in human kinesin and therefore the mechanism seems to be conserved among conventional kinesins. This has not been described so far, and the structural basis has not been elucidated yet.
To locate residues involved in signaling the neck-linker position, we exchanged Tyr80, Asn81, and Asn298, and tested the effect in the cross-linkable monomeric AE-mutant background. All three triple mutants alleviated the almost complete inhibition that the AE cross-link alone produced. The strongest effect was seen in the AE-Y80G mutant, possibly because tyrosine offers multiple ways of interaction. Remarkably, Tyr80 is almost absolutely conserved as a tyrosine or phenylalanine among motile kinesins but is absent in members of the non-motile Kinesin-13 subfamily, emphasizing the importance of this residue for motility. Our data give hints why Tyr80 is so important. First, observations on the triple mutant AE-Y80G indicate that Tyr80 plays a central role in communicating the docked neck-linker position to the catalytic core. The primary effect may be to induce low microtubule affinity in the docked neck-linker state and, possibly as a consequence, to slow ADP release. Secondly, the slow gliding velocity of the Y80G single mutant (1.0 μm/s) points at a thermodynamic role in neck-linker docking. A similar behavior has been described for the neck-linker residues Val331/Asn332 in human kinesin (corresponding to residues 336/337 in NcKin) (9). Replacing these residues with alanine caused a motility defect and abolished the small favorable change in free energy for neck-linker docking. The aberrant gliding behavior of the Y80G mutant is likely to have a similar cause. To explain the uncoupling phenotype in the cross-linked protein background however, the efficiency of the docking process is irrelevant. In the present study the neck-linker is fixed in its docked position and thus the kinetics of the docking process cannot interfere with measurements under oxidizing conditions. On the other hand the formation of disulfide bridges is not prevented by unfavorable thermodynamic conditions, as demonstrated by the lack of motility. In gliding assays microtubules bound to the oxidized triple mutant but did not move, indicating that the kinesin population is virtually fully cross-linked. As observed for the AE mutant, any uncross-linked fraction would probably cause at least slow gliding.

The data described so far hint at a model, according to which a conformational flexibility of the neck-linker and a feedback via the interface to the motor core are required for normal enzymatic activity. How does the docked neck-linker state inhibit the motor activity?

### TABLE 6

| Construct          | ADP     | AMP-PNP |
|--------------------|---------|---------|
|                    | DTT     | DTNB    | DTT     | DTNB    |
|                    | μM      | μM      | μM      | μM      |
| NcKin-343          | 0.52 ± 0.16 | 2.00 ± 0.14 | 0.25 ± 0.11 | 0.18 ± 0.04 |
| A226C/E339C        | 0.58 ± 0.19 | 1.04 ± 0.09 | 0.22 ± 0.10 | 0.41 ± 0.06 |
| NcKin-436hKT       | 1.05 ± 0.44 | 0.79 ± 0.00 | 0.25 ± 0.06 | 0.53 ± 0.16 |
| S3C/A334C          | 0.22 ± 0.07 | 0.32 ± 0.01 | 0.19 ± 0.08 | 0.44 ± 0.23 |

To locate residues involved in signaling the neck-linker position, we exchanged Tyr80, Asn81, and Asn298, and tested the effect in the cross-linkable monomeric AE-mutant background. All three triple mutants alleviated the almost complete inhibition that the AE cross-link alone produced. The strongest effect was seen in the AE-Y80G mutant, possibly because tyrosine offers multiple ways of interaction. Remarkably, Tyr80 is almost absolutely conserved as a tyrosine or phenylalanine among motile kinesins but is absent in members of the non-motile Kinesin-13 subfamily, emphasizing the importance of this residue for motility. Our data give hints why Tyr80 is so important. First, observations on the triple mutant AE-Y80G indicate that Tyr80 plays a central role in communicating the docked neck-linker position to the catalytic core. The primary effect may be to induce low microtubule affinity in the docked neck-linker state and, possibly as a consequence, to slow ADP release. Secondly, the slow gliding velocity of the Y80G single mutant (1.0 μm/s) points at a thermodynamic role in neck-linker docking. A similar behavior has been described for the neck-linker residues Val331/Asn332 in human kinesin (corresponding to residues 336/337 in NcKin) (9). Replacing these residues with alanine caused a motility defect and abolished the small favorable change in free energy for neck-linker docking. The aberrant gliding behavior of the Y80G mutant is likely to have a similar cause. To explain the uncoupling phenotype in the cross-linked protein background however, the efficiency of the docking process is irrelevant. In the present study the neck-linker is fixed in its docked position and thus the kinetics of the docking process cannot interfere with measurements under oxidizing conditions. On the other hand the formation of disulfide bridges is not prevented by unfavorable thermodynamic conditions, as demonstrated by the lack of motility. In gliding assays microtubules bound to the oxidized triple mutant but did not move, indicating that the kinesin population is virtually fully cross-linked. As observed for the AE mutant, any uncross-linked fraction would probably cause at least slow gliding.

The data described so far hint at a model, according to which a conformational flexibility of the neck-linker and a feedback via the interface to the motor core are required for normal enzymatic activity. How does the docked neck-linker state inhibit the motor activity?
The primary effect of neck-linker docking seems to be a reduction of microtubule affinity that was observed in co-sedimentation assays with ADP (Fig. 5), but also in competitive gliding assays with cross-linked and wild-type NcKin (Fig. 4). The higher $K_{D, MT}$ values might also be caused by a lower microtubule affinity, although a lower degree of processivity could also explain the effect. Remarkably, according to available Kinesin-1 crystal structures the neck-linker docks in the vicinity of the primary microtubule-binding domain (30, 34). Therefore, it is well possible that the transition from a weak to a strong binding state is disturbed by neck-linker docking.

Which other steps might be affected? As a consequence of aberrant microtubule interaction, the ADP release might be blocked. Assays on the dimeric AE mutant pointed in that direction, but we were unable to confirm this effect on monomeric constructs. Alternatively, the catalytic properties of the kinesin ATPase might be hindered in general. We actually observed a significant decrease of the basal ATPase activity (2- and 5-fold for the dimeric and monomeric AE mutant, supplemental table). However, the decrease was much smaller than the microtubule-dependent effect. This indicates that the docked neck-linker mainly decreases the microtubule affinity.

This may also be the case in the natural docked state. According to standard models, stepping is a consequence of ATP binding to the microtubule-bound head and the subsequent very rapid neck-linker docking process. This conformational change enables the other head to bind to a forward microtubule site, and leads to an intermediate, possibly strained dimer configuration, where the two heads are forced apart. Cryo-EM models demonstrate that the tip of kinesin (loop 10) points toward the microtubule plus end, and thus predict that the neck-linker of the trailing head is forced into its docked conformation in the intermediate where both heads are microtubule-bound. Possibly, the rear head of this intermediate behaves similarly to the docked constructs investigated in this study.

It should be noted, however, that there are a number of structural unknowns in this model. Although comparison of different crystal models does not predict larger conformational changes involved in disulphide formation than the structural variability of neck-linker positions present in different structure models (estimated 0–8 Å), the exact nature of the introduced cross-links is unresolved. For structural investigations, we tried to isolate the kinesin-microtubule complex with cross-linked neck-linker. As observed in co-sedimentation assays, the affinity of kinesin with permanently docked neck-linker is low, and therefore a reasonable degree of decoration was not possible. We therefore were unable to compare the state we captured in the cross-linked AE mutant with that observed previously (8). Here, the microtubule-bound ADP form of kinesin was found to contain a non-docked neck-linker conformation, whereas the present study hints at the importance of a kinesin-ADP complex with docked neck-linker and low microtubule affinity. These apparently contradictory views might be reconciled by the existence of different ADP states that differ in structure and kinetic behavior. Alternative ADP states have been assumed before to interpret presteady state kinetic experiments, and have been suspected to show different microtubule binding affinities. One might speculate that the microtubule-bound ADP-state imaged by Rice et al. (8) and the one inferred here are in direct chemical equilibrium.

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