Engineering a Lever into the Kinesin Neck*

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To probe for a lever arm action in the kinesin stepping mechanism, we engineered a rodlike extension piece into the tail of rat kinesin at various points close to the head-tail junction and measured its effects on the temperature dependence of velocity in microtubule gliding assays. The insert comprised two contiguous α-actinin triple-coil repeats and was predicted to fold into a stiff rodlike module about 11 nm long. The effects of this module were greater the closer it was placed to the head-tail junction. When inserted distal to the head-tail junction, at Asn639 in the dimeric K9340GST, the insert had no effect. When inserted closer to the heads at Val576 into K93376GST, the insert slowed progress below 22 °C but accelerated progress to ~125% of wild type above 22 °C. The most dramatic effect of the synthetic lever occurred when it was inserted very close to the headneck junction, at Glu439 into the single-headed construct K9340GST. This construct was immotile without the insert, but motile with it, at about 30% of the velocity of the dimeric control. The α-actinin module thus confers some gain-of-function when inserted close to the head-neck junction but not when placed distal to it. The data exclude the presence of a lever arm C-terminal to Val576 in the kinesin tail but suggest that a short-throw lever arm may be present, N-terminal to Val576 and contiguous with the head-neck junction at Ala339.

Kinesin molecular motors have twin motor domains, or “heads,” and “walk” along microtubules (MTs), touching down with alternate heads to binding sites spaced 8 nm apart along the microtubule protofilament axis. It is unknown if walking occurs in tightrope fashion along a single microtubule protofilament or whether kinesin can straddle between two protofilaments. But in either case, the two heads must be coordinated such that each can alternately detach and search for a binding site, while the other remains attached and acts as a holdfast. Runs of 100 or more steps are typical of unloaded kinesin (1, 2), indicating efficient coordination between leading (free) and trailing (holdfast) heads. Some degree of guidance or conformational restriction is presumably applied by the trailing head to the leading head, such that the leading head binds to the correct site on the microtubule, in the productive direction and in line, so as to maintain its characteristics accurately axial tracking (3). At the same time, the free head needs a considerable degree of conformational freedom to allow it to stretch and attach to the new binding site, transiently developing the “bridge” (both heads attached) structure that defines walking (4–6).

At the head-tail junction, the two kinesin heads are joined at their C termini to a rodlike tail built predominantly from α-helical coiled coil. The original crystal structure of the monomeric human kinesin head in complex with MgADP (7) had as its most C-terminal feature the helix α6, the extreme C terminus of the head being disordered and therefore invisible. The more recent two-headed crystal structure of rat kinesin (8), also in complex with MgADP, reveals the head-tail junction in more detail. The coiled coil tail starts at Ala339. Between the end of α6 and Ala339, the chain folds into two short β-strands, β9 and β10. C-terminal to Ala339, and beyond the region visible in the crystals, the coiled coil structure of the tail is predicted to be interrupted by Pro/Gly-rich insertions, which may trigger local unwinding and strand separation of the tail. Strand separation has been proposed as a possible basis for the ability of the molecule to bridge between adjacent binding sites on the MT (5, 10); there is, however, no direct evidence for it. Studies on isolated peptides tended, on the contrary, to indicate that two-chain dimers were remarkably stable (11, 12), but the possibility of local unwinding nonetheless remains (13). More recently, mutation of the neck region of kinesin in an attempt to stabilize the coiled coil structure of the kinesin neck did not affect the ability of the molecule to walk (step repetitively) between binding sites (14).

Presumably, the relatively complex structure of the region surrounding the head-tail junction of kinesin (5) reflects a requirement for this region to somehow satisfy conflicting conformational demands: it must be flexible enough to facilitate the diffusional search made by the leading head for its imminent binding site and yet stiff enough to support strain as the kinesin molecule exerts traction force on the microtubule. Recent work has revealed that attachment of the head of ncd, a MT minus end-directed motor, to the tail of nkin, a kinesin-like plus end-directed motor, can force the ncd head to reverse its normal direction of movement and move toward MT plus ends (15, 16). It is thus clear that the structure of the head-tail attachment can profoundly influence the action of the motor. On the other hand, Inoue et al. (17) have reported rapid and efficient motion of bead-kinesin complexes along MTs when the kinesin heads were attached to the beads via a flexible linker fused in at residue 340, which suggests that structuring of the region immediately C-terminal to 340 is unnecessary for efficient movement. Relevant also are experiments from the Gelles laboratory, in which it was shown that kinesin single heads fused at residue 340 to a biotinylated protein drive MT sliding only slowly (0.09 μm s−1) compared with the typical rate for longer, dimeric constructs (0.75 μm s−1; 26 °C) (18).

Broadly, two kinds of physical mechanism have been proposed for the cross-bridge action of molecular motors: those based on directionally biased capture by the track of a thermally diffusing cross-bridge (19) and those based on force-producing conformational changes that occur in the cross-
bridge following attachment to the track (20). A schematic diagram exemplifying the potential involvement of these two sorts of process in the stepping action of kinesin is shown in Fig. 1. The tethered diffusion of the leading head occurs prior to capture by the microtubule and is relatively insensitive to the presence or absence of a lever arm. The tilting and subsequent conformational change that may follow attachment to the microtubule will have their effective amplitude amplified by any lever arm.

For both kinesin and myosin, the general problem of the molecular motor mechanism is to understand the contribution of these two processes and specifically the protein-structural pathway via which the turnover of nucleotide in the motor active site drives the mechanical cycle. The lever arm theory for myosin action relates to the force-producing conformational change type of mechanism, whereby following binding of the myosin head to its actin track, a force-producing conformational shift occurs which is amplified by the swinging action of a lever arm that is rigidly attached to the head (21). For kinesin, the relative importance in the mechanism of these two types of process, rectified diffusion and conformational change, is not currently clear, but for the related myosin motor, the need for some sort of structured lever arm at the head-tail junction is more evident. There is evidence that the neck region (the light chain binding domain) of myosins acts as a mechanical amplifier, a semi-rigid cantilever arm, which swings through an arc of several tens of degrees in response to conformational changes within the head which are in turn driven by the ADP and/or P, release steps of ATP turnover (21). In experiments to test this idea, the putative lever arm of myosin has been lengthened mutagenically, by inserting an artificial rod repeat (22) or extra copies of the wild-type light chain binding domain (23). Both experiments produced an increase (respectively 1.3-fold for a doubling of lever length and 2.6-fold for a 3-fold increase in lever length) in unloaded sliding velocity. In the case of the Uyeda et al. experiments (23), the increase was linearly proportional to the predicted lever arm length. A lever arm mechanism thus seems plausible in the myosin case, although definitive proof is still being sought (24).

The experiments described here are loosely modeled on those described above for myosin. We probed for a lever arm action in kinesin by extending the presumptive lever arm (the region of the tail closest to the head) using protein engineering. If a lever arm action is involved in the kinesin mechanism, then lengthening this region should accelerate the motor in proportion to the total length of the lever arm, as was observed for myosin. In seminal work, Goldstein and colleagues (41) coupled the kinesin head to part of the rodlike tail of Drosophila spectrin, making the junction at residue 448 of Drosophila kinesin, and showed that the chimera was active in MT sliding assays. In the current experiments, we worked with kinesin-glutathione S-transferase (GST) fusions in which the tail was truncated to various extents, close to the head-tail junction now shown to be at Ala139. We inserted into these a rigid extension piece consisting of copies of the putatively rigid α-actinin rod domain and compared the performance of the truncated constructs with and without the inserted α-actinin module. For the dimeric constructs, this insertional mutagenesis produced maximally only a relatively modest increase in velocity (125% of wild type), despite an estimated 11-nm increase in predicted lever arm length (see below).

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant Kinesins**

**Kinesin-GST Fusions**—The expression vector used for this study for production of kinesin-GST fusion and for kinesin-α-actinin-GST fusions was a pET17b (Novagen)-based bacterial vector that had been modified by inserting a PCR-amplified GST sequence at the EcoRI restriction site of the multilinking site of the vector. The vector fuses the GST protein C-terminally to the various motor tubulins, which were inserted between the NdeI and EcoRI sites (5). All PCR reactions were performed according to Lockhart et al. (38).

Kinesin inserts corresponding to amino acids 1–340, 1–376, and 1–401 were PCR-amplified from the original rat kinesin heavy chain clone (a kind gift of Dr. Scott Brady and Ann Sperry) with 5′-CGGCTC-TCATATGCGGAGACCACATGCAGC-3′ as the forward primer and the following reverse primers: for K340, 5′-ACCTCTGAAATCTGC- CTTAGTTC-3′ (producing a 1029-base pair fragment); for K376, 5′-ACCGGAAGATTCACAGCTTCCGTTCT-3′ (producing a 1128-base pair fragment); for K401, 5′-ACCCCGGAGTTGTTCTATGAT- GGTTGTGT-3′ (producing a 1203-base pair fragment). The forward primer introduced an NdeI site at the 5′-end of the kinesin. The reverse primers added a SacI site at the 3′-end of the truncated C termini of the kinesins. After amplification, all of the kinesin fragments were cut with NdeI/SacI and were ligated into pET17b vector (Novagen), which had been cut with NdeI/SacI. Following ligation, the constructs were digested with SacI/RI in preparation for ligation to the α-actinin insert. The α-actinin insert was PCR-amplified from the original chick embryonic α-actinin clone (27) using 5′-ACCGAGCTCGAGATCCGGAGGCTA-GAGGCTG-3′ as the forward primer and 5′-AATCCGGAGATTCTCTTCGAGACCTGCG-3′ as the reverse primer. The forward primer introduced a SacI site, and the reverse primer introduced an EcoRI site. The amplified insert corresponded to amino acids 385–615 of the α-actinin clone, the two central repeats of the rod. The insert was cut with SacI/R1 and ligated to each of the three above-described SacI/RI-cut pET17b kinesin plasmids. Following ligation, these plasmids were again digested with NdeI/R1 to excise the α-actinin-kinesin fusions and ligated into the above described NdeI/RI-cleaved pET17b GST vector. The expression of the SacI site introduced two extra amino acid codons (corresponding to EL) at the kinesin-α-actinin junction.

**Preparation and Polymerization of Tubulin**

Tubulin was prepared from porcine brain as described by Lockhart and Cross (28). Microtubules were assembled by the addition of MgCl₂ to 2 mM and GTP to 1 mM, incubated at 37 °C for 30 min, and stabilized by adding taxol to 10 μM.

**Expression and Purification of Recombinant Constructs**

For expression of the recombinant proteins, a single colony of freshly transformed competent BL21(DE3) cells was grown overnight to saturation in liquid 2× YT medium (with 100 μg/ml ampicillin). The next morning, the overnight culture was diluted 1:50 into prewarmed 2× YT (Difco) medium and grown to an A₅⁶₀ of 1.0. The culture was then cooled to 22 °C over a period of 30 min with shaking and subsequently induced with 0.8 mM isopropyl-1-thio-β-D-galactopyranoside and shaken for a further 4 h at 22 °C. Cells were harvested by centrifugation, and the cell pellets were quickly frozen in liquid nitrogen. The cell pellets were stored in a −70 °C freezer.

For preparation of the protein, the thawed pellets were resuspended in phosphate-buffered saline containing additionally 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol (GST column buffer), supplemented with Complete Protease Inhibitor tablets (Boehringer Mannheim) (1 tablet/30 ml of buffer). For more labile proteins, extra leupeptin and aprotinin were added. The cell suspension was incubated on ice with 0.8 mM isopropyl-1-thio-β-D-galactopyranoside and shaking for 10 min on ice. The cell-free supernatant was obtained by centrifugation at 27,000 × g.

This supernatant was incubated with 5–7 ml of glutathione-Sepharose 4B beads (Pharmacia Biotech, UK) equilibrated with GST column buffer, on a roller at 4 °C for 30–40 min, and the beads were then packed into a column. The beads were first washed with 10 volumes of...
the GST column buffer and then 5 volumes of Hi-Trap Q column buffer, comprising 50 mM PIPES, pH 6.9, 5 mM MgCl$_2$, 1 mM EGTA, 50 mM NaCl, and 1 mM dithiothreitol.

The bound protein was eluted with 20 mM GSH, pH 7.0, in Hi-Trap Q column buffer, and the pooled eluate was loaded onto a 1-ml Q column at a rate of 1 ml/min. Hi-Trap Q chromatography was performed with an FPLC system (Pharmacia). The column was washed with 10 volumes of Q column buffer and then 5 volumes each of 0.1 M, 0.2 M, and 0.3 M NaCl in Q column buffer. Most of the protein eluted at 0.2 M NaCl. The purity of the eluted protein was analyzed by SDS-polyacrylamide gel electrophoresis, and peak fractions were pooled; mixed with 15% glycercol, 0.2 mg/ml of o-casein, and 100 μM ATP; frozen, and stored in liquid nitrogen in small aliquots. The concentrations of the purified expressed proteins were determined spectrophotometrically (29). Proteins stored this way were active indefinitely. Once thawed, the protein aliquots were used immediately and never refrozen.

**Gel Filtration Chromatography of the Constructs**

Analytical gel filtration chromatography was done in sample storage buffer supplemented with 200 mM NaCl, 1 mM dithiothreitol, 10 μM ATP using a Superose 6 column, and a 500-μl sample loop. The column was ran at 0.2 ml/min, and the absorbance of the eluate was monitored at 280 running the recorder chart at 0.5 cm/ml. 0.2-ml fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis.

**Motility Assay for Recombinant Constructs**

Motility assays for all of the constructs were carried out according to Ref. 30 in PEMD buffer (80 mM PIPES, 1 mM EGTA, 2 mM MgCl$_2$, 5 mM dithiothreitol). 0.2 mg/ml o-casein (Sigma) was added to the protein samples as soon as they were thawed. 10–15-μl flow cells were constructed from uncleaned glass coverslips supported on two strips of Scotch Magic tape and coated using casein-containing motor at concentrations of 10–30 μg/ml at room temperature (normally 23 °C) for 5–6 min. The flow cells were then rinsed with PEMD buffer freshly supplemented with 1 mM ATP, followed by perfusion with a suspension of 1 μM MT in the same buffer supplemented with 20 μM taxol. After a few seconds, the flow cell was rinsed with PEMD plus 1 mM ATP and mounted on the microscope stage. The microscope was an upright Nikon Microphot SA with standard DIC transmission optics and a Nikon 60 × 1.4 NA DIC objective, illuminated using light from a 100-watt mercury arc lamp fed through a fiber optic light scrambler (Technical Video, Woods Hole). At the input end of the scrambler, the light was filtered by reflection from a dichroic mirror to remove infrared and at the output end by a green interference filter. The DIC slider was set close to extinction, and the image was further magnified using an in line zoom ocular to produce a 22 m square field at the CCD camera faceplate. The video signal was real-time back-ground-subtracted and contrast-enhanced using a Hamamatsu Argus 20, and then recorded simultaneously to SVHS video tape and (in time lapse) to a PC hard disc via a frame grabbing card controlled from the RETRAC package (see Ref. 30). The temperature of the specimen was clamped by flowing temperature-controlled water through soft copper tubes, which were wrapped around the objective and stage. The microscope and stage were wrapped in cling film to restrict air flow over the specimen, and the actual temperature of the flow cell was monitored by insertion of a thermocouple probe. For each temperature assayed, a new flow cell was prepared. For each data point, at least three different 20-frame clips, each from a different field, were grabbed. Tracking was done using the RETRAC package as described previously (5). RETRAC calculates an updated S.D. of the mean velocity for each additional x, y data point, and tracking was continued (more fields were analyzed) until this value stabilized. In the velocity versus temperature plots, the error bars for this S.D. fall within the data points.

**RESULTS**

**Phasing and Insertion of the o-Actinin Spacer**—The current experiments were modeled on the earlier work on myosin lever arm extension by Anson and colleagues (22), in which part of the rodlike central domain of o-actinin was used as the lever arm extension. It is clear from recent electron microscopy and from a series of solution studies (32–34) that the four central 106-residue pseudorepeats of o-actinin fold up into four quasi-independent “beads” within a rodlike domain. The detailed pattern of backfolding of the primary chain into the four presumed spectrin-like triple coiled coil structural repeats is unknown but is presumed to be similar to that of spectrin (35), for which a crystal structure is available (36). It is believed for o-actinin that the antiparallel dimerization of the intact o-actinin molecule is due to the interaction of pseudorepeats 1 and 4. We used the central pseudorepeats 2 and 3 (using the optical repeat boundaries defined by Critchley (33, 34, 37)) of chick embryo o-actinin, on the grounds that these should form a stable, elongated, freely soluble and non-self-associating module. This rodlike module corresponds approximately to the Dictyostelium o-actinin module used by Anson et al. in the myosin experiments (22), allowing the two sets of data reasonably to be compared. The correspondence is not exact, however; the work by Anson et al. (22) used repeat 1 or repeat 1 plus 2 of Dictyostelium o-actinin, whereas here we have used repeat 2 plus 3 of chick o-actinin. The module used here was confirmed in FPLC gel filtration experiments to be soluble and non-self-associating when bacterially expressed as a C-terminal fusion to GST (not shown). The guest o-actinin module was inserted into the kinesin neck at three points, as shown in Fig. 2A; the sequences of the kinesin-o-actinin junctions are shown in Fig. 2.

**Association State of Truncation/Insertion Mutants**—The purity of the expressed, purified proteins was checked using SDS microslab electrophoresis and is shown in Fig. 3. The physical state of association of the proteins was examined using analytical gel filtration. We were particularly concerned to check for combined effects of truncation of the kinesin and insertion of the o-actinin spacer module on the aggregation state of the motors. Previous work has shown that K340 is predominantly monomeric and that K401 is predominantly dimeric (38). These constructs have been used in our EM reconstruction work (39, 40), which allowed direct visual confirmation that K340 is monomeric and that K401 is dimeric. Fig. 4 shows FPLC analytical gel filtration data for the current truncation/insertion mutant constructs.
insertion mutants. The K340AAGST mutant, which inserts the \( \alpha \)-actinin module adjacent to the heads (Fig. 2), is seen to be predominantly monomeric, with a major monomer peak corresponding to the mobility of K340GST (not shown) and a secondary peak corresponding to a smaller population of dimers. K430GST, with no \( \alpha \)-actinin insert, consists predominantly of dimers, with smaller amounts of probably monomers and tetramers. The truncation/insertion mutants K376AAGST and K401AAGST are dimeric, with a variably sized minor leading shoulder, which may correspond to a small population of tetramers, formed by the antiparallel dimerization of the GST fusion protein. We conclude that the insertion of the \( \alpha \)-actinin spacer does not substantially influence the intramolecular dimerization equilibria of the constructs, consistent with our prediction based on the earlier findings of Critchley and co-workers (33, 34) that the central two \( \alpha \)-actinin repeats are not involved in the dimerization of \( \alpha \)-actinin chains.

Effects of Truncating the Kinesin Tail—As previously reported for K430GST (5), kinesin-driven MT sliding velocity was in general steeply temperature-dependent. Fig. 5 shows a comparison of the performance of a control recombinant construct, K430GST, with that of a series of truncation and truncation/insertion mutants. Progressive truncation of region 340–430 of the tail, which separates the kinesin heads from the GST fusion protein (Fig. 2) had little effect on the temperature dependence of sliding velocity until the tail became very short, at which point (fusion to GST at kinesin residue Glu340) the protein was monomeric, and motility ceased (not plotted). The Arrhenius energies for sliding for the three constructs K430GST, K401GST, and K376GST had very similar values (Table I). That truncation of the tail between Val376 and Lys430 has little effect on MT gliding velocity itself suggests that a lever arm element is absent in this region of the kinesin tail. Likewise, since the K376GST construct deletes the flexible Pro/Gly-rich insert proposed to promote strand separation of the tail, we can conclude that this element also has no effect on gliding velocity. That the monomeric K340GST was immotile in the current assays is unsurprising. Kinesin truncated close to this point and N-terminally fused to GST was previously reported to be extremely sluggish in surface sliding assays, running at about 1⁄400 of the dimeric GSTK430 rate (41). Kinesin monomers fused C-terminally to a biotinylated protein at kinesin residue 340 are also very slow (18), moving at about 10% of the rate of dimers. It was suggested in both cases that this was because the proximity of the motor domains to the glass surface restricted their conformational freedom, and the same thing was proposed for truncations of the kinesin-related motor ncd (42).

Effects of \( \alpha \)-Actinin Insertion Module Depend on Insertion Site—Insertion of the rodlike \( \alpha \)-actinin spacer module into these various truncation mutants had differing effects on performance, depending on the site of insertion (Figs. 5 and 6). Insertion of the spacer at 401 into the K401GST truncation had little effect, the behavior of both K401GST and K401AAGST being similar to that of the K430GST dimeric control. The addition of the \( \alpha \)-actinin spacer module to K376GST to form K376AAGST generated biphasic Arrhenius behavior (Fig. 5). K376AAGST ran more slowly than wild type below about 22 °C but ran more quickly, at about 125% of control, above 22 °C. The effect is striking; at the transition point, velocity doubles from 0.4 to 0.8 m s\(^{-1}\) with a 2 °C increase in temperature. The origin of this transition from inhibition to activation of sliding is not clear. Biphasic Arrhenius plots typically indicate a temperature-dependent conformational change, and perhaps the transition may correspond to temperature-dependent release of the \( \alpha \)-actinin moiety from a state in which it is bound to other molecules or to the glass
substrate. However, this is speculation. What is clear is that the insertion of the extension module N-terminal to the predicted random region at −376–401 produced a measurable gain-of-function over wild type.

The most dramatic effect of the spacer module occurred for the nonmotile monomeric K3340GST construct, which became motile upon insertion of the spacer module. The velocity of K3340AAGST was very strongly temperature-dependent, and Arrhenius plots revealed markedly biphasic behavior (Fig. 6). The maximum velocity measured, 0.22 μm s⁻¹ at 27 °C, is by far the fastest velocity so far observed for MT sliding on a surface of monomeric kinesin heads. The phase transition in this case was nonetheless from a low to a high activation energy with increasing temperature, indicating that above the transition temperature, progress was inhibited to some extent. Below we discuss possible explanations for the gain-of-function effects of the α-actinin insert on the monomeric K3340GST and on the much faster dimeric K376AAGST. Table I summarizes the data on Arrhenius energies.

**DISCUSSION**

Possible mechanisms of movement of kinesin along microtubules have been reviewed by Howard (4). As discussed in the Introduction and diagrammed in Fig. 1, two kinds of general physical mechanism have been proposed for the action of molecular motors, those based on directionally biased capture by the track of a thermally diffusing cross-bridge (19) and those based on force-producing conformational changes that occur in the cross-bridge following attachment to the track (20). The general mechanistic problem is to understand the contribution of these two processes and specifically the protein structural pathway via which the turnover of nucleotide in the motor active site drives the mechanical cycle. The lever arm theory for myosin action relates to the conformational change type of mechanism, whereby following binding of the myosin head to its actin track, a force-producing conformational shift of the head occurs, which is amplified by the swinging action of a rigidly attached lever arm (21).

It is now very clear that kinesin moves processively (walks) along microtubules, the mechanical and chemical action of its two heads being coupled by an as yet unknown mechanism, such that (at least) detachment of the trailing head is inhibited until secure attachment of the leading head has occurred. The mechanical coupling may also inhibit futile turnover of ATP, since only about one ATP is usually consumed per step (6). Several other kinesin family members appear less processive (5), which may relate to their lacking the Pro/Gly-rich region found adjacent to the heads in the tail of kinesin proper. Most recently, it was shown that connecting the heads of ncd (a minus end-directed kinesin homologue) to the tail of nkin (plus end-directed) drives the ncd heads to move toward the plus end (15, 44). The structure and mechanics of the head-tail junction thus can profoundly affect function, specifying the direction of progress and possibly the degree of processivity of the motor.

The lever arm model predicts that the length of a lever in the region of the head-tail junction should dramatically affect the velocity of the motor. At low loads, a roughly linear relationship is expected between lever arm length and sliding velocity, because in a lever arm mechanism the velocity of sliding is determined predominantly by the range and velocity of the lever arm motion, and at low loads the velocity of lever arm motion should be maximal. A linear relationship is only expected if the lever arm is essentially rigid (45), but such a linear relationship was indeed observed in surface sliding assays when the lever arm of myosin was extended by adding extra copies of the light chain binding domain (23). In the current experiments, we first asked if truncation of the section of the tail C-terminal to the head-tail junction can affect performance. Progressive truncation of the tail from 430 to 376 had essentially no effect. We conclude that a lever arm is absent from this region. An earlier report did describe a progressive reduction in MT sliding velocity on truncating within this region (41), but there are several differences in the experiments: we used rat kinesin instead of Drosophila; we used purified protein instead of GST minicolumn eluates; we used C-terminal GST fusions instead of N-terminal; and, perhaps most important, we included casein in the assays, which has been shown to work as a lubricant, reducing internal loads, which can slow down sliding (1). The most extreme truncation we made, to 340, produced monomers instead of dimers and stopped MT sliding. The earlier Stewart et al. (41) experiments, did detect sliding by a GSTK339 construct, but velocity was profoundly reduced by 420-fold over the dimeric control, and again these data relate to an N-terminal rather than a C-terminal GST fusion.

Our second set of experiments asked whether an added lever
arm, inserted at the truncation sites, can produce a gain of function. The α-actinin module we used as a synthetic lever is predicted to be about 11 nm long, assuming its two repeats are half the length of the intact four-repeat central rod of α-actinin, determined recently by EM to be about 22.5 nm long (31). Insertion of this module at Asn401 into K401GST had no effect on performance. The region 376–401 is Pro/Gly-rich and predicted to be flexible (9, 11, 12), and if so it is not surprising that insertion of a rigid element C-terminal to a flexible element has little effect. By contrast, insertion of the α-actinin module at Val376 to produce K376AAGST did affect performance, reducing velocity below 22 °C and increasing velocity above 22 °C to about 125% of wild type. Inserting the α-actinin lever into the short monomeric K340GST construct also produced a gain-of-function, activating movement of this otherwise inactive construct, albeit only to a level of about 30% of the velocity of the dimeric K376AAGST. We thus find that inserting the synthetic lever distant from the head-tail junction has no effect but that inserting it close to the head-tail junction does produce a gain of function. How are we to interpret this?

Previous work on the surface sliding assay with native kinesin and with recombinant kinesins has emphasized the importance of geometry and surface chemistry as determinants of sliding velocity. MT sliding velocity is similar for assays done on surfaces of multiple dimeric motors and for assays done with single dimeric (wild-type) kinesin molecules (1, 46) but is reported to be progressively reduced as the motor is truncated. At low loads in the sliding assay, sliding velocity will be affected

**Fig. 5. Motility assay data.** Top, truncation constructs. Bottom, truncation plus insertion constructs. The raw data are shown fit by least squares to the Arrhenius equation $V = Ke^{Ea/RT}$, where $V$ is the sliding velocity, $K$ is a pre-exponential factor, $E_a$ is the Arrhenius energy, $R$ is the gas constant, and $T$ is the absolute temperature. The smaller plots are the more traditional Arrhenius plot, which graphs $1/T$ versus $\ln V$, and they can be fit by least squares to a straight line of slope $E_a/R$ and offset $\ln K$. The dotted line in the K376AAGST display is an overlay of the fit to the K376GST data. Data points above 305 K were ignored in the fitting procedures, since a fall in performance above 305 K was noted and ascribed to thermal denaturation.
by the conformational freedom of the heads. There is direct evidence for this; as mentioned above, for kinesin (41) and its oppositely directed homologue ncd (42), sliding velocity is progressively reduced as the tail of the motor is progressively truncated, and the heads are forced to operate closer to the surface. The qualities of the surface are also important; blocking unproductive binding of the motor to the glass surface by adding casein has been shown to be crucial for MT sliding assays at low kinesin concentrations (1). In the current high density assays, we adsorbed very high concentrations (tens of micromolar) of kinesin in the presence of casein, so that (presumably) casein and kinesin are competing for the glass surface. The measured sliding velocities were constant and reproducible, both moment-to-moment and for different batches of purified motors on different days, and on this basis we believe that the glass surface was evenly and completely coated with a lawn of motor and that the measured properties are those of such an active surface of close-packed kinesin. It is known that in surface sliding assays driven by single wild type kinesin molecules, the stepping action of the two heads remains coupled, allowing MTs to slide several μm (equivalent to hundreds of steps) over a single kinesin molecule before detaching. In this situation, the rate of MT sliding is set by the rate of the repetitive mechanochemical cycle of the single working kinesin molecule. The head-head coupling is such that the MT is tethered to the surface by the trailing head of a pair until the leading (free) head locates and binds securely to its imminent site: the sooner the site can be found, the sooner the MT can slide forward. It is reasonable to assume that head-head coupling also exists in multimotor assays, although the situation is more complicated, with multiple motors interacting with each microtubule and each motor presumably experiencing impulses of force deriving from the others.

Where head-head coupling exists, the rate of MT sliding depends directly on the rate of alternation between the heads. (The situation is different for myosin, where each head detaches from the track after a single mechanical cycle, and the time taken for it to recover is irrelevant (19).) In the kinesin case, the rate of head-head alternation might be limited by the time taken for diffusion to capture by the free head or by the time taken for a notional power stroke following a force-generating conformational change by the forward head. The two possibilities correspond to the two types of mechanochemical model under consideration. The increased velocity of the K376AAGST construct implies a decreased cycle time, which might be due to the insert reducing the diffusion-to-capture time for the free head, or to its increasing the amplitude of a lever arm effect. In a pure lever arm model, the diffusional component of cycle time is regarded as negligible, and velocity is linearly dependent on lever arm length. This criterion is clearly not met: we detected only a 25% increase in MT sliding velocity due to lever arm insertion, for a predicted 11-nm increase in lever arm length, amounting to roughly twice the combined length of the head plus the section of the tail between Glu340 and Val376 (Fig. 2). We can therefore safely rule out a pure (myosin like) rigid lever arm model for kinesin and infer, therefore, that rectified, tethered diffusion is the major contributor to the step distance and that the inserted α-actinin extension piece accelerates the motor by reducing the time taken for diffusion to MT capture by the leading head. Importantly, while inserting the α-actinin module at Val376 does accelerate the motor, simply having a longer length of the authentic kinesin tail present does not, suggesting that the insert needs to be stiff or at least partially stiff to be effective. This would be consistent with a role for the insert in restricting and focusing the search pattern of the head.

We have argued that the major contributor to the step distance is rectified diffusion, but might a residual lever arm action still be present, outside of the region probed by the current experiments? Our view is that this a very real possibility. Any contribution by a short throw lever arm action, meaning a directional conformational change amplified by a stubby lever, would be expected to occur after attachment of the leading head to the microtubule and while the trailing head is still attached to the MT (Fig. 1). Formation of this both-heads-attached (bridge) conformation of the motor would probably require some unzipping of the neck region coiled coil (although there is no direct evidence for this) and would translate the bifurcation point of the tail in the productive direction, exerting plus end-directed force on the trailing head and triggering its release from the MT. There is direct electron microscopic (47) and proteolytic (48) evidence for nucleotide-dependent conformational changes of MT-attached heads. To amplify these, the postulated stubby lever would need to be stiffly joined to the head-neck junction at Ala339. A striking result of the current work is the activation of the motility of K340 by the α-actinin insertion. This shorter construct is monomeric and therefore must lack the intramolecular head-head coordination present in the longer dimeric construct, although intermolecular coordination must still be present, as discussed recently by Hancock and Howard (49). This loss of coordination

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**TABLE I**

Arrhenius energies for MT sliding driven by the various constructs

| Construct   | Arrhenius energy (KJ mol⁻¹ K⁻¹) | Arrhenius energy (KJ mol⁻¹ K⁻¹) |
|-------------|---------------------------------|---------------------------------|
| K340GST     | 60.9                            | 60.8                            |
| K340I       | 61.5                            | 67.0                            |
| K340AAGST   | 58.1                            | 69.1                            |
| K376        | 53.5                            | 56.8                            |
| K376AAGST   | 91.5/71.9                       | 100.9/72.6                      |
| K340AAGST   | 87.3/150                        | 61.8/169                        |

*Unweighted fit to raw data.

a Linear fit to log-inverse plot.
means that the single headed Kα340AAGST construct runs at about 20% of the rate of Kα376AAGST, but once this loss of efficiency is allowed for, we find that Kα340AAGST moves quickly, much faster than previous values for single-head-driven sliding and about 50% faster than the “anatomically correct” construct recently described by Hancock and Howard (49), which has one head attached to a full-length two-chain coiled coil tail. The apparent functional improvement, if real, suggests that the stiff α-actinin module, coupled in at Glu340, could be potentiating the action of a short throw lever, which is contiguous with the head-tail junction, and perhaps also act as a tension transducer to improve the coupling between distant molecules in the sliding assay. This view of the mechanism as a combination of short throw lever arm and long range rectified diffusional scanning offers a possible explanation for the otherwise puzzling data of Inoue et al. (17), who showed that kinesin heads attached to beads via a flexible linker fused in at residue 340 could move the beads at wild-type rates. The linker would facilitate diffusional scanning but leave intact the action of the postulated stubby lever, N-terminal to, and contiguous with, residue 340.

In summary, the data allow us to exclude the presence of a lever arm and the utility of an introduced lever arm in the region Val376-Lys430 of the kinesin tail but leave open and to some extent support the possibility that a short throw lever arm may operate N-terminal to Val376 and contiguous with Ala339. Mutations around the head-tail junction create problems in the MT gliding assays because of proximity to the surface, and further progress will require the use of single molecule optical trapping.

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