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The conformational cycle of kinesin

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The stepping mechanism of kinesin can be thought of as a programme of conformational changes. We briefly review protein chemical, electron microscopic and transient kinetic evidence for conformational changes, and working from this evidence, outline a model for the mechanism. In the model, both kinesin heads initially trap Mg·ADP. Microtubule binding releases ADP from one head only (the trailing head). Subsequent ATP binding and hydrolysis by the trailing head progressively accelerate attachment of the leading head, by positioning it closer to its next site. Once attached, the leading head releases its ADP and exerts a sustained pull on the trailing head. The rate of closure of the molecular gate which traps ADP on the trailing head governs its detachment rate. A speculative but crucial coordinating feature is that this rate is strain sensitive, slowing down under negative strain and accelerating under positive strain.

Keywords: kinesin; microtubule; ncd

1. INTRODUCTION

Two-headed ukinesin (ubiquitous kinesin, kif5b) molecules are able to step processively along microtubules against an applied load (Howard *et al.* 1989). Elegant single-molecule mechanical measurements have shown that the motor steps 8 nm at a time, corresponding to the axial separation between tubulin heterodimers in a microtubule protofilament, and stalls at a retroactive force of about 6 pN (Svoboda *et al.* 1993; Svoboda & Block 1994; Kojima *et al.* 1997). Recent reports suggest that progress is tightly coupled to ATP consumption, approximately one ATP molecule being consumed per 8 nm step (Schnitzer & Block 1997; Hua *et al.* 1997; Coy *et al.* 1999; Cross 1997*a*).

At low loads, the kinesin stepping mechanism can be thought of as a programme of discrete conformational changes, each reaction step in ATP processing producing modest motions in the active site which are amplified so as to drive the motor into a new conformation. Under load, the motor tries to execute this same programme of conformational changes, but the rate constants for some or all of the transitions are altered by physical tension. The reciprocal relationship between the tension on the motor molecule and its active site chemistry is called the mechanochemical coupling. To elucidate the mechanism of mechanochemical coupling, we need to know the minimum energy conformation of the motor for each different nucleotide occupant of the active site and the rate constants for transitions between these conformations at low load, together with the influence of external strain on the structure and on these rate constants.

We have studied several kinesin superfamily members in relation to conformation and the kinetics of conformational changes. Here, we briefly discuss current evidence for conformational changes in the kinesins, and suggest that the various lines of evidence point to a general model for mechanochemical coupling, in which the key coordinating process is the ADP-trapping reaction which precedes detachment. The rate constant for this step is postulated to be strain dependent, and hence is expected to both buffer and broker internally generated and externally applied forces, and to provide a basis for head–head coordination in processive stepping. The model is testable and is supported by preliminary evidence.

2. CLASSES OF CONFORMATIONAL STATE

Like myosin, conformational states of kinesins can usefully be classified as weak (low affinity, tending to detach) and strong (high affinity, able to hold force). All kinesin family members so far examined show a common pattern whereby the nucleotide-free state binds most tightly, and analogues of intermediates in ATP processing weaken the binding in order of effectiveness ADP > ADP·AlF_4 > AMPPNP. Data for various kinesin family members are summarized in table 1. The ADP state is always the weakest binding.

3. CRYO-ELECTRON MICROSCOPY OF KINESIN AND NCD

Beautiful X-ray structures of the ADP conformations of several kinesin family monomers (Sablin *et al.* 1996; Kull *et al.* 1996; Gulick *et al.* 1998; Sack *et al.* 1997) and dimers (Kozielski *et al.* 1997; Sablin 1998) have been obtained, but we do not yet have atomic-resolution views of the other conformational intermediates in nucleotide turnover. Thus far, all attempts to crystallize kinesins in states other than

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Table 1. *Dissociation constants for two-headed recombinant enzymes*

(Note buffers and temperatures vary slightly between studies. Dimeric constructs only are listed because there is evidence that head–head interactions may affect values.)

	apyrase	K·ATP	K·ATPgS	K·AMPNP	K·ADP·Pi	K·ADP·AlF_4	K·ADP·BeF_4	K·ADP
rat kinesin K401 (Crevel *et al*. 1996)	0.003	12.4	4.2	0.3	—	1.8	—	11.3
human k413 (Rosenfeld *et al*. 1996)	—	—	—	—	17	—	0.5	7
Drosophila ncd (Crevel *et al*. 1996)	0.2	8.5	2.3	0.2	—	5.5	—	12
Drosophila ncd (Song & Endow 1998)	—	5.8	—	—	—	—	—	5.3
Xenopus eg5 (Lockhart & Cross 1996)	1.1	>10	7.7	0.02	—	—	—	>10
Saccharomyces cerevisiae kar3 (Song & Endow 1998)	—	1.6	—	—	—	—	—	1.7

K·ADP have failed (J. Kull, personal communication). Electron microscopy (EM) offers the possibility to visualize these crystallographically intractable conformations in complex with microtubules, and hence to probe for changes in shape and attitude of the motor as the nucleotide in the active site is exchanged. For kinesin even the weakest binding state, the motor·ADP state, has a micromolar dissociation constant, and so can be populated at workable protein concentrations and visualized in the EM.

Early work using negative staining of single heads indicated a plus-end directed tilt of microtubule-attached kinesin heads on ADP release (Hirose *et al*. 1995). Negative staining can in principle deliver very high resolution, but because of worries that the staining process can damage the specimen, subsequent work has been done using rapid freezing of unstained specimens. Using cryo-electron microscopy (cryo-EM) and helical reconstruction procedures, Hirose and Amos have obtained three-dimensional electron-density maps of the ADP, rigor and AMPPNP conformations of two-headed recombinant rat ukinesin and *Drosophila* ncd attached to microtubules, at between 2 and 4 nm resolution (Hirose *et al*. 1996, 1998, 1999). Comparing the conformational cycles of these two motors is of particular interest because ncd moves towards the minus-end of microtubules, whereas ukinesin moves toward the plus-end. For each motor, substantial conformational differences are seen between the different nucleotide states. In all cases, only one of the two heads binds to the microtubule, the other being held clear of the microtubule surface, poised to probe for its next site. The tethered heads are posed in different positions in the different conformations. Figure 1 shows reconstructions of two-headed kinesin and two-headed ncd in three different nucleotide states.

In all three conformational states of ncd, the tethered head is held to the right of the bound head and points towards the minus-end of the microtubule. In the ADP state it is held at roughly the same level as the bound head, in an arrangement which is similar but apparently not identical to that in the N·ADP dimer crystal structure (figure 2*g*,*h*). In the nucleotide-free state, the junction between the heads has moved up and a little to the right, and the tethered head has moved outwards from the microtubule surface. In the AMPPNP state, assumed to mimic the ATP state, the tethered head moves about 2 nm towards the plus-end, is retracted somewhat against the bound head, and twists clockwise about 10°.

For kinesin, changing the nucleotide in the active site has a more substantial effect on the position of the tethered head. In ADP, the tethered head is held above and to the left of the bound head in an arrangement that mimics that in the dimeric K·ADP crystal structure (figure 2*c*,*d*). In apyrase, the tethered head moves down and lies against the bound head, pointing towards the minus-end with its long axis roughly parallel to the microtubule axis. In AMPPNP, the tethered head shifts upwards and over to the right of the bound head. The apparent mass of the tethered head is reduced in the ADP and AMPPNP states, suggesting mobility. Dynamics produce blurring, which reduces the apparent mass. This problem does not seem to affect ncd, suggesting its free head may be less dynamic. Mandelkov and co-workers (Hoenger *et al*. 1998; Thormahlen *et al*. 1998) do not see the second head mass at all in their reconstructions of kinesin, and this could be due to dynamics (as we believe), or to binding of both heads to the microtubule in their specimens (as they believe). Both heads certainly can bind at least transiently, but the kinetic work shows that binding of the second head in AMPPNP is about tenfold slower than first-head binding (Ma & Taylor 1997; Gilbert *et al*. 1998), so that at saturating concentrations of kinesin, one would predict that most sites would become occupied by first heads.

The attached heads of kinesin and ncd look very similar, and bind to identical sites on tubulin, consistent with earlier work showing mutually competitive binding (Lockhart *et al*. 1995). Conformational differences between the bound heads in different nucleotide states can nonetheless be seen. In particular, a spike on the left side of the bound head of ncd shifts upwards towards the plus-end on AMPPNP binding, as was first noted in the negative staining work on kinesin single heads (Hirose *et al*. 1995). A protrusion at top right of the kinesin·ADP-bound head is missing in the apyrase state. For both motors, nucleotide-free and AMPPNP conformations of the bound heads sit closer to the microtubule surface and have a larger contact interface than do the ADP conformations, consistent with tighter binding.

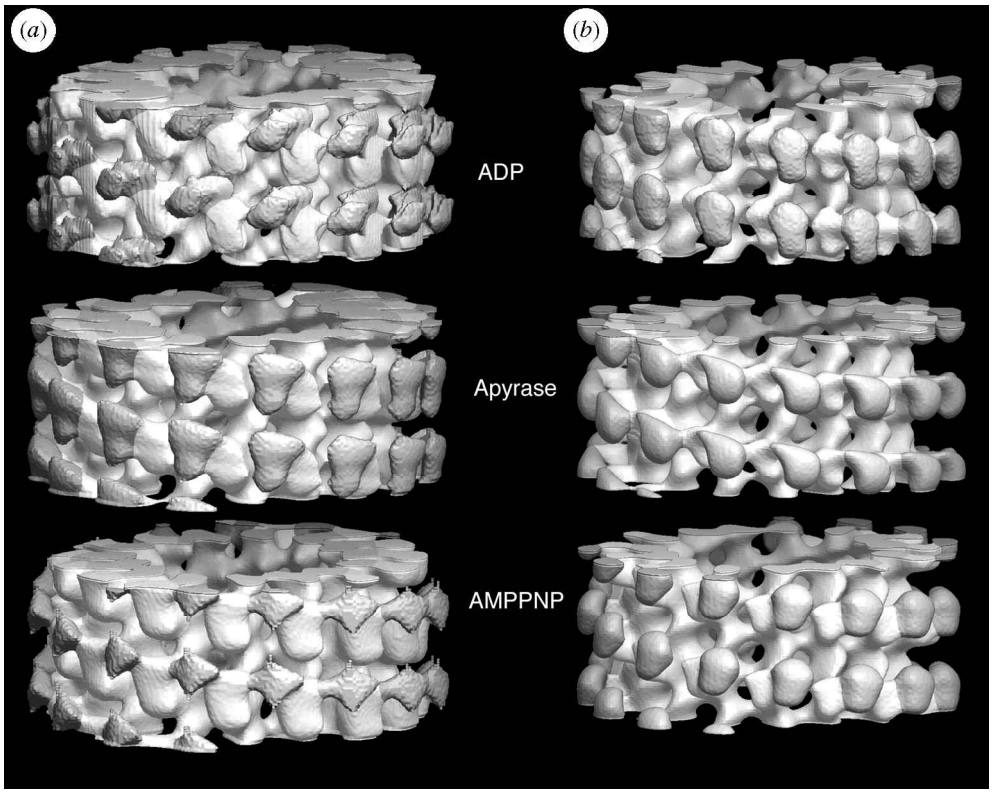

Figure 1. Comparison of three-dimensional cryo-EM reconstructions of three different nucleotide states of (*a*) dimeric *Drosophila* ncd and (*b*) rat ukinesin. Microtubule plus-ends are towards the top of the figure.

The cryo-EM results reported by Arnal & Wade (1998) are broadly similar to our own, with the interesting difference that in their experiments the ukinesin·ADP conformation looks closer to the ukinesin·AMPPNP reconstruction of Hirose *et al.* (1995). We believe this may indicate that two different ADP states can occur, corresponding to different points in the conformational cycle.

4. MAPPING THE MICROTUBULE-BINDING INTERFACE

In parallel with fitting studies, we and others have directly probed for the microtubule-binding surface of kinesin using biochemical techniques. Woehlke *et al.* (1997) made an alanine scan of charged residues on the candidate microtubule-binding face of human kinesin. Charged residues are distributed broadly over the candidate binding surface. Of those residues whose mutation to alanine had an effect, substitution of positive residues reduced the K_m (Michaelis constant) for microtubule activation of the ATPase, whilst substitution of negative residues had the opposite effect. We took an alternative approach, using proteolytic protection to probe for the microtubule-binding interface of kinesin and ncd monomers, and to search for nucleotide-induced conformational changes (Alonso *et al.* 1998). The data are shown in figure 2. We identified a set of 18 cleavage sites in kinesin and 19 in ncd, and asked which were protected from proteolysis by microtubule binding. A substantial surface is protected by microtubule binding (figure 2). Similar surfaces are protected on kinesin and on ncd, in agreement with earlier work which showed competitive binding of ncd and kinesin heads to microtubules (Lockhart *et al.* 1995), and with recent fitting studies (Hirose 1999) that suggest the bound heads of kinesin and ncd are congruent. Using the conventional orientation (figure 2), the protected surface is on the opposite side of the molecule to that containing the ATP-binding site.

5. FITTING X-RAY MAPS INTO ELECTRON MICROSCOPY MAPS

In the case of ncd, Sosa *et al.* (1997) fitted the ncd·ADP monomer coordinates into an ncd·AMPPNP EM structure. The junctions between the heads can be seen clearly, and were used as the main constraint on the fitting. Because the tethered head of ncd does not appear to move very much between different states, this yielded an orientation which agreed with the alanine scanning and proteolysis data. Attempts to fit kinesin in the same orientation as ncd produced unacceptable steric clashes for dimeric kinesin, indicating that something was wrong, as noted by Mandelkow and colleagues (Hoenger *et al.* 1998; Thormahlen *et al.* 1998).

The problem turns out to be that all this fitting was done using motor·ADP crystal structures, and motor·AMPPNP EM maps. Our ukinesin·ADP EM map turns out to be very different from the ukinesin·AMPPNP EM maps (Hirose 1999). A fit made by Kozieslki *et al.* (1998) to the K·ADP EM map of Arnal & Wade (1998) places sites which are protected from proteolysis in an exposed position, and cleavable sites in a protected position, and hence is inconsistent with the proteolysis data. However, fitting of the ukinesin·ADP dimer crystal structure into our newly obtained ukinesin·ADP cryo-EM map produces a different and more satisfactory result.

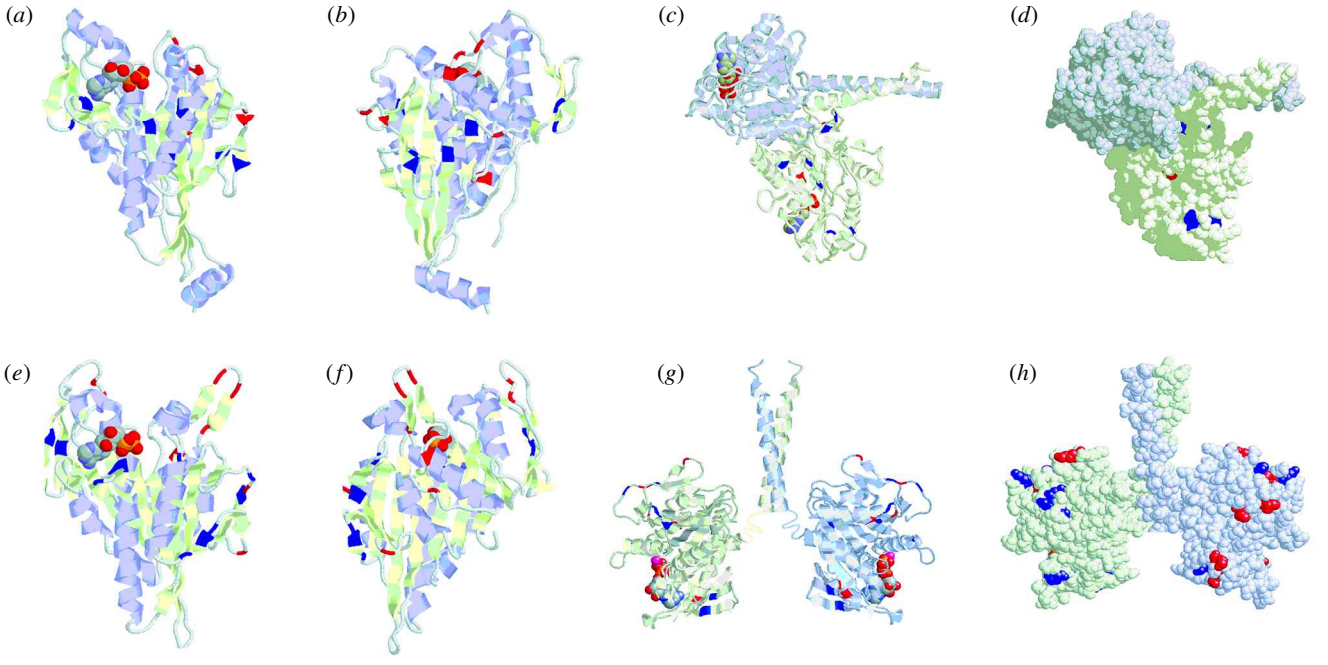

Figure 2. Front and back cartoon views of (*a*, *b*) rat ukinesin·ADP monomers and (*e*, *f*) *Drosophila* ncd·ADP monomers in the conventional orientation (Kull *et al*. 1996) showing proteolytic sites protected (red) and unprotected (blue) by microtubule (MT) binding. (*c*, *d*) Cartoon and spacefilling views, respectively, of the newly fitted orientation of kinesin·ADP dimers when bound to MTs. The view is face-on to the MT surface, which would be in the plane of the page, with the plus-end towards the top of the page, as in figure 1. (*g*, *h*) The same for ncd·ADP dimers.

Since the heads are shaped like slightly flattened hearts, the flattening can be used to find the best rotational attitude of the tethered heads. The new fit (Hirose 1999) is still consistent with the alanine scan and proteolytic data but, being rotated by about 60° from the original position, avoids the steric clashes. It also brings the nucleotide-binding site nearer to the microtubule and, interestingly, puts loop L9, which is thought to be analogous to a G-protein's switch I loop (Vale & Fletterick 1997), into direct contact with tubulin.

6. NUCLEOTIDE-INDUCED LOOP MOVEMENTS

The proteolytic study described above identified several sites which were protected from proteolytic cleavage by a change of nucleotide in the active site, in the absence of microtubules. Three sites, I170 and L154 in rat kinesin, and Y622 in ncd, were cleavable with ADP in the active site, but not with AMPPNP in the site. Possibly the L8 and L12 loops which contain the latter two residues are displayed so as to mediate the initial kinesin–microtubule binding interaction, but are then retracted to allow the switch to a strong binding conformation. Another possibility is that the tail of the molecule lies over these sites with AMPPNP in the sites, and then moves aside when ADP is in the site. Probe work is also beginning to reveal molecular motions (Naber *et al*. 1997).

7. KINETICS OF CONFORMATIONAL TRANSITIONS

ADP trapping by kinesin was first described by Hackney (1988), who also was the first to show that mixing two-headed ukinesin with microtubules released only half of the bound ADP, so that it was necessary to add a chase of ATP to release the other half (Hackney 1994). Subsequent work showed that AMPPNP could also chase ADP from the second head, and it was suggested that in the normal mechanical cycle, ATP binding to the bound (trailing) head is the trigger for microtubule binding by, and consequent microtubule-activated ADP release from, the leading head (Ma & Taylor 1997; Gilbert *et al*. 1998). We recently tried analogous experiments with the *Neurospora* kinesin (nkin), which moves around fourfold faster than conventional (brain) ukinesin. The *Neurospora* enzyme is mechanically processive, as was evident from single-molecule optical-trapping experiments. Kinetic studies showed, however, that compared with brain kinesin, AMPPNP binding to the trailing head was relatively inefficient at triggering release of leading-head ADP. ATPγS and ATP induced much faster second-head ADP release, and we accordingly concluded that for nkin, hydrolysis was necessary for rapid second-head ADP release. Importantly, both the ukinesin and nkin data would be consistent with a single model in which progressive acceleration of leading-head microtubule binding occurs as ATP is processed on the trailing head. For both kinesin and nkin, ADP binding to the trailing head induced only slow leading-head ADP release, indicating that the spontaneous detachment of the trailing head K·ADP is too slow to be on the normal pathway, and suggesting the need for facilitated detachment.

8. TOWARDS A MECHANOCHEMICAL MODEL

Figure 3 shows a mechanochemical scheme which derives from the evidence outlined above. In the scheme, the motor generates force and gains some ground by changing its conformation (its shape or tilt angle) after attachment to the track, but most of the per-head step distance of 16 nm is gained by facilitated diffusion of

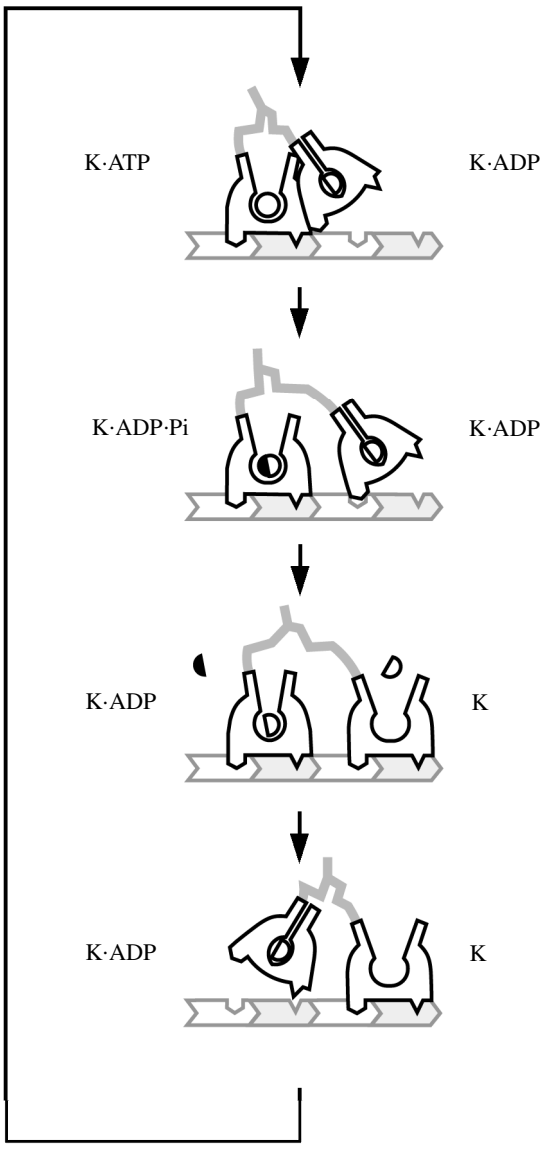

Figure 3. Mechanochemical scheme. Binding and hydrolysis of ATP (open circle) to the back foot accelerates front foot attachment and associated microtubule-activated ADP (open semicircle) release. A resulting pull on the back foot accelerates the ADP-trapping reaction which precedes detachment, possibly by accelerating Pi (filled semicircle) release.

tethered heads prior to microtubule binding (Peskin & Oster 1995). Tethered heads are assumed to diffuse randomly about a null point defined by the connection to the bound head, but have the ability to recognize and lock in productive diffusional excursions by binding stereospecifically to the track and changing conformation. Reversing the directional bias of this diffusion-to-capture process would provide a straightforward way to reverse directionality in two motors which otherwise underwent similar conformational changes once bound to the track (Lockhart & Cross 1994). In this way it is possible to rationalize the finding that the direction of ncd heads can be reversed by splicing them to a kinesin tail (Henningsen & Schliwa 1997; Case *et al*. 1997), and the inverse experiment reversing kinesin (Endow & Waligora 1998; Cross 1997*b*). The conformational change accompanying landing (that corresponding to microtubule-activated release of trapped ADP) can contribute to the step distance, and this contribution might be amplified via a lever-arm action, though evidence to date suggests that for ukinesin at least, any such lever must be relatively short (Inoue *et al*. 1997; Romberg *et al*. 1998; Mazumdar *et al*. 1998).

It is clear that in the absence of microtubules, both heads trap ADP. Collision of this complex with microtubules releases ADP from one of the two heads, but not from the other. The processive cycle starts at this point (figure 3), with one head in rigor, and the other trapping ADP. ATP binding and hydrolysis then occur on the trailing head, and these progressively facilitate the diffusional search made by the tethered (leading) head for its next site, perhaps by shifting the null point (the head–head junction or neck) closer to the new site, or perhaps by releasing the tethered (leading) head from a state in which it is bound to the other head. Once the leading head locates its site, it binds and releases its ADP, concomitantly exerting a sustained pull on the trailing head. This pull accelerates closure of the gate which traps ADP on the trailing head, and the trailing head rapidly detaches.

Considerable uncertainty surrounds the inorganic phosphate (Pi) release step. The K·ADP·AlF_4 data suggest that K·ADP·Pi is a tightly bound state, and that Pi release could therefore do work. This is an attractive idea because in myosin, Pi release is associated with a large free energy change that probably drives a lever-arm motion. In the kinesin case we speculate that Pi release is also strain dependent, but there are no data. For the purposes of the present discussion, were Pi release to be strain dependent (slowing down under negative strain and accelerating under positive strain), the net effect would be the same: the rate of formation of the detaching trapped-ADP conformation would be strain dependent. This postulated strain dependence of ADP trapping would provide a mechanism for coordinating neighbouring heads, because opening of the gate which traps ADP on the leading head is synchronized with closure of the equivalent gate on the trailing head. For non-processive kinesins (we believe ncd and eg5 are essentially non-processive (Crevel *et al*. 1997; Stewart *et al*. 1998)), this type of mechanochemical scheme can still apply, but tension signals are passed between heads via the microtubule. The key feature of the scheme, strain-sensitive ADP trapping, works equally well as a coordinating mechanism when the heads are not part of the same molecule. Mechanical processivity within the ukinesin subfamily would then be a result of structural adaptations which allow dimeric motors to bridge between neighbouring binding sites, whilst retaining essentially the same strain-dependent chemistry as other family members.

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