Unusual properties of the fungal conventional kinesin neck domain from *Neurospora crassa*

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Fungal conventional kinesins are unusually fast microtubule motor proteins. To compare the functional organization of fungal and animal conventional kinesins, a set of C-terminal deletion mutants of the *Neurospora crassa* conventional kinesin, NcKin, was investigated for its biochemical and biophysical properties. While the shortest, monomeric construct comprising the catalytic core and the neck-linker (NcKin343) displays very high steady-state ATPase \( k_{\text{cat}} = 260 \text{s}^{-1} \), constructs including both the full neck and adjacent hinge domains (NcKin400, NcKin433 and NcKin480) show wild-type behaviour: they are dimeric, show fast gliding and slower ATP turnover rates \( k_{\text{cat}} = 60–84 \text{s}^{-1} \), and are chemically processive. Unexpectedly, a construct (NcKin378, corresponding to *Drosophila* KH381) that includes just the entire coiled-coil neck is a monomer. Its ATPase activity is slow \( k_{\text{cat}} = 27 \text{s}^{-1} \), and chemical processivity is abolished. Together with a structural analysis of synthetic neck peptides, our data demonstrate that the NcKin neck domain behaves differently from that of animal conventional kinesins and may be tuned to drive fast, processive motility.

*Keywords:* ATPase kinetics/domain analysis/ fungal kinesin/processivity/synthetic peptide

**Introduction**

Conventional kinesins from fungi are microtubule-dependent motor proteins that presumably play a role in the intracellular transport of secretory vesicles. In comparison with their relatives from animals, they move ~3-fold faster (Kirchner et al., 1999; Steinberg, 2000). Despite this distinctive feature, fungal kinesins move processively in a stepwise fashion, like their animal counterparts (Crevel et al., 1999). The analysis of their core motor domains reveals a high degree of sequence conservation with conventional kinesins from human, rat and fly. Consequently, fungal kinesins can be considered as natural mutants of their animal counterparts, which makes them interesting models for studying motor function. The conventional kinesin NcKin from *N. crassa* was the first fungal kinesin described and therefore the prototype of this group of kinesins (Steinberg and Schliwa, 1995). Today, similar kinesins from other ascomycetes, basidiomycetes and zygomycetes are known (Lehmler et al., 1997; Grummt et al., 1998a; Wu et al., 1998; Requena et al., 2001).

Like animal conventional kinesins, their fungal relatives consist of several domains (Vale and Fletterick, 1997; Woehlke and Schliwa, 2000). The conserved core motor domain, which is responsible for the catalytic activity, is located at the N-terminus, and is linked to the so-called neck domain via a short neck-linker that seems to be important for the generation of motility (Rice et al., 1999). Therefore, the shortest construct in this study (NcKin343) comprises the motor core and the entire neck-linker, the so-called 'head'. In animal kinesins, the neck forms a two-stranded α-helical coiled-coil, causes dimerization of the motor molecule and joins the motor domain to the flexible, extended hinge region (Seeberger et al., 2000; Woehlke and Schliwa, 2000). The domains further C-terminal, the stalk and tail domains, seem to provide the cargo-binding site and a mechanical linker to the functional motor domain, and have been shown not to alter the motile behaviour *in vitro* (Grummt et al., 1998b; Seiler et al., 2000).

The functional organization of fungal conventional kinesins has been inferred from studies on the *Syncephalastrum racemosum* kinesin (Grummt et al., 1998b). These studies suggest that regions outside the core motor domain play important roles in determining the velocity and processivity of the motor. Thus, deletions of the neck and hinge domains, either alone or in combination, cause a dramatic decrease in the gliding velocity accompanied by an increase in the ATP turnover. Interestingly, replacement of the fungal hinge by the corresponding domain from the *Drosophila melanogaster* kinesin heavy chain, DmKHC, restores the gliding velocity but still leaves one significant parameter altered: the chemical processivity.

The ability to move processively is a feature of kinesin motors and refers to the fact that a single motor molecule can remain attached to its substrate filament during several mechanical steps. This behaviour can be observed in single-molecule microscopic assays, but is also reflected in kinetic properties (Hackney, 1995). In an ATPase assay, a processive motor appears to possess a lower half-maximal activation constant for microtubules \( K_{0.5, \text{MT}} \) than expected from its binding properties because the re-binding process necessary to initiate a new catalytic cycle
Fig. 1. Domain structure of kinesin. The location of domains relevant for this study is indicated in the top part of the figure. Below, the sequence alignment of three fungal (upper sequences) and three animal conventional kinesins highlights the differences of fungal and animal kinesins. In the core motor domain and the neck-linker, the homology, as indicated by asterisks for amino acid identity and dots for similarity, of all representatives is obvious. The central parts of the neck domains clearly differ between fungal and animal kinesins. In the hinge, all representatives diverge significantly. The position of the truncations used for this study is indicated above the alignment. For comparison, published Drosophila kinesin truncations are marked below (Jiang et al., 1997). The positions of the peptides Kα1 and Kα3 are indicated above the alignment. Domain boundaries were derived from the dimeric rat crystal structure (Kozieleski et al., 1997) and coiled-coil prediction programs for the hinge and coil 1 domains (Lupas, 1996). The phase of the neck coiled-coil is indicated above the sequence alignment. Kinesins from: NcKin, N.crassa (SwissProt accession No. L47106); UmKin2, Ustilago maydis (U92845); SrKin, S.racemosum (AF225894); MmKin, Mus musculus (X61435); HsKHC, Homo sapiens ubiquitous kinesin (X65873); DmKHC, D.melanogaster (M24441).

Results
To address the question of whether fungal conventional kinesins possess the same functional domain organization as their animal relatives, we expressed a series of C-terminally truncated versions of the N.crassa conventional kinesin, NcKin (Steinberg and Schlwa, 1995). The ends of these constructs were chosen to (i) fit domain boundaries as closely as possible (Kozieleski et al., 1997) and (ii) be comparable with published Drosophila kinesin mutants (Jiang et al., 1997). Figure 1 shows a sequence alignment of fungal and animal kinesins indicating the positions at which the mutants of this study terminate. As only two-headed conventional kinesins are known to possess processive motility (Hancock and Howard, 1998), the dimerization characteristics of NcKin were investigated.

Oligomerization studies
To map the regions responsible for dimerizing NcKin, the sizes of the deletion mutants were determined (Table I). According to the gel filtration and sucrose density gradient experiments, the short motor versions NcKin343, 378 and 383 are monomeric; constructs longer than 390 residues are dimeric. By analogy with animal kinesins, the neck domain was expected to dimerize the heads. Hence, the failure of NcKin378 and 383 to dimerize came as a surprise (Figure 1). The NcKin378 protein ends at a residue homologous to DmKHC381, which clearly is dimeric (Figure 1; Jiang et al., 1997). It is unlikely that the C-termini were degraded since we also obtained this result with the C-terminally cysteine-tagged versions and were still able to label the tags.

As dimerization is a quantitative process, we estimated the dimerization constant, K_d. In the sizing experiments, at least 8 μM kinesin was used, yet no signal for a dimeric
form of NcKin378 could be detected. We therefore conclude that the $K_d$ for dimerization of NcKin378 lies significantly above 8 μM. To test whether ionic conditions prevented dimerization of NcKin378, the gel filtration was performed in the presence of 75 and 200 mM KCl. However, the elution volume relative to the standard proteins was unchanged, demonstrating that NcKin378 remains monomeric under low salt conditions. It was not possible to test even lower ionic strengths because of interactions of the Sephadex matrix and the sample. The slightly longer NcKin383, in contrast, showed a second peak in the gel filtration in some preparations. This peak corresponded to the lighter and contained <10% of the total protein, as determined by ATPase rates. Accordingly, the dissociation constant $K_d$ is expected to range above 300 μM under these experimental conditions (38 μM NcKin, 200 mM KCl). In comparison, DmKHC381, which resembles NcKin378, dimerizes below 1 μM, as a construct comprising amino acids 1–413 (Rosenfeld et al., 1996; Jiang et al., 1997). From these studies, we conclude that the NcKin neck domain has a much lower capability to dimerize the motor heads compared with animal kinesins.

The NcKin391 construct is only eight amino acids longer than NcKin383 and clearly forms stable dimers under all salt conditions tested, and all longer NcKin versions also form dimers. To test whether the hinge domain alone is able to dimerize, a mutant of NcKin433 lacking the neck was investigated. This Δneck construct is monomeric, indicating that a combination of neck and hinge sequence motifs acts cooperatively to dimerize NcKin in solution.

**Peptide studies**

To elucidate the structural basis of the aberrant dimerization behaviour, synthetic peptides derived from the neck domain were investigated. The similarity of animal and fungal kinesin neck domains is low in its N-terminal part but becomes obvious in the C-terminal 12 amino acids (Figure 1). Algorithms predict a low coiled-coil-forming propensity for the N-terminal part of the animal kinesin neck due to unfavourable, bulky or charged residues in the coiled-coil positions a and d. In contrast, peptide studies showed that the neck does form a stable two-stranded α-helical coiled-coil, which was later confirmed by the dimeric rat kinesin crystal structure (Koziełski et al., 1997; Mori et al., 1997; Tripet et al., 1997). For the fungal neck, the prediction indicates coiled-coil formation (Lupas, 1996), but experimental evidence has been lacking so far.

We therefore synthesized a NcKin neck domain peptide comprising amino acids 338–379 (peptide Kn1, Figure 1), which exceeds the expected coiled-coil by five amino acid residues at both the N- and C-termini. The solubility and conformational state of this peptide were found to be strongly pH dependent (Figure 3A). Only under acidic conditions (pH 3), the circular dichroism (CD) spectrum resembled those usually reported for two-stranded α-helical coiled-coils ($\Theta_{222}/\Theta_{208} = 1.03$). This observation suggests an important role for the ionized glutamic acid residues for the onset of this type of fold. Moreover, at pH 3, the molar ellipticity $\Theta_{222}$ decreases sigmoidally from ~6000 to ~19000° cm$^2$/dcm, with peptide concentrations rising from 10$^{-6}$ to 10$^{-4}$ M, indicating formation of intermolecular associations (data not shown). In agreement with this interpretation, ESI-MS spectra clearly reveal masses that can be assigned to peptide dimers (Supplementary figure 1, available at The EMBO Journal Online; Li et al., 1993; Przybilski and Glöckler, 1996).

Evidence that this interaction is due to coiled-coil formation was obtained from 2,2,2-trifluoroethanol (TFE) titration experiments. TFE is known to stabilize secondary structures, but to disrupt higher order structures such as coiled-coils (Soennichsen et al., 1992; Bodkin and Goodfellow, 1996). The ratio $\Theta_{222}/\Theta_{208}$ was found to change in a sigmoidal manner from 1.03 in phosphate buffer, pH 3, to 0.96 in the presence of 70% TFE, indicating a cooperative transition from a two-stranded α-helical coiled-coil to a single-stranded α-helix (Figure 3B). To stabilize the dimeric form of the neck peptide, a second, slightly shorter peptide was used, which was cross-linked via a disulfide bridge at the a position of the first heptad repeat by replacing the endogenous proline with cysteine (Kn3, Figure 1). Taking the ellipticity ratio $\Theta_{222}/\Theta_{208}$ as an index for coiled-coil formation, the dimeric peptide Kn3 was found to fold into this type of conformation at neutral pH (50 mM phosphate buffer pH 7). The slightly lower ellipticity ratio of ~1 compared with Kn1 (1.03) could result from the constraints introduced by the disulfide bridge.

To determine the stability of the NcKin neck coiled-coil, thermal denaturation of the longer, wild-type-sequence peptide Kn1 was performed. Thermal unfolding of the peptide proceeds cooperatively with a $T_m$ of 47.2°C, and an isodichroic point at 203 nm, which is indicative of a

<table>
<thead>
<tr>
<th>Construct</th>
<th>$r_{Stokes}$ (nm)</th>
<th>Density ($S_{20, w}$)</th>
<th>Derived mass (kDa)</th>
<th>Predicted mass (kDa)</th>
<th>Oligomerization state</th>
</tr>
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<tbody>
<tr>
<td>NcKin343</td>
<td>2.8 ± 0.1 n = 2</td>
<td>3.3 ± 0.2 n = 2</td>
<td>38</td>
<td>37.4</td>
<td>monomer</td>
</tr>
<tr>
<td>NcKin378</td>
<td>2.9 ± 0.2 n = 2</td>
<td>3.5 ± 0.2 n = 2</td>
<td>42</td>
<td>41.9</td>
<td>monomer</td>
</tr>
<tr>
<td>NcKin383</td>
<td>3.6 ± 0.1 n = 2</td>
<td>3.7 ± 0.0 n = 2</td>
<td>55</td>
<td>42.5</td>
<td>monomer</td>
</tr>
<tr>
<td>NcKin433-Δneck</td>
<td>4.3 ± 0.1 n = 2</td>
<td>3.3 ± 0.0 n = 2</td>
<td>58</td>
<td>44.2</td>
<td>monomer</td>
</tr>
<tr>
<td>NcKin391</td>
<td>4.8 ± 0.1 n = 2</td>
<td>4.6 ± 0.2 n = 2</td>
<td>90</td>
<td>43.4</td>
<td>dimer</td>
</tr>
<tr>
<td>NcKin400</td>
<td>4.2 ± 0.1 n = 2</td>
<td>4.1 ± 0.2 n = 2</td>
<td>71</td>
<td>43.8</td>
<td>dimer</td>
</tr>
<tr>
<td>NcKin433</td>
<td>4.7 ± 0.1 n = 3</td>
<td>4.3 ± 0.1 n = 2</td>
<td>83</td>
<td>47.2</td>
<td>dimer</td>
</tr>
<tr>
<td>NcKin480</td>
<td>5.0 ± 0.1 n = 1</td>
<td>4.2 ± 0.2 n = 6</td>
<td>87</td>
<td>52.8</td>
<td>dimer</td>
</tr>
<tr>
<td>NcKin928</td>
<td>5.6 ± 0.1 n = 5</td>
<td>9.3 ± 0.3 n = 4</td>
<td>215</td>
<td>105.0</td>
<td>dimer</td>
</tr>
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</table>
two-state transition from a folded to an unfolded state (Figure 4). During the recoling process, however, formation of β-structure takes place, thus preventing the extraction of thermodynamic parameters. Due to the low solubility of the Kn1 peptide, related NMR conformational analyses could not be carried out. Similarly, attempts to purify a bacterially expressed fragment comprising the NcKin neck and hinge domains also failed due to solubility problems.

These results suggest that the neck domain is required but not sufficient for NcKin dimerization. Its unfavourable protonation state at physiological pH prevents folding into a two-stranded α-helical coiled-coil. Additional stabilizing motifs present in hinge residues 384–400 apparently facilitate helix and/or coiled-coil formation by an unknown mechanism.

To characterize the impact of dimerization and C-terminal deletions on motility, ATPase and processivity, functional assays were performed (Table II).

**Motility assays**

Motility assays were performed on streptavidin-coated coverslips with biotin–maleimide-labelled motors, derived from the cysteine-tagged protein versions. Unlabelled motor proteins did not adhere to the glass surface by themselves. Since the ATPase activities of truncated *Neurospora* kinesins with a C-terminal cysteine tag were indistinguishable from those of the corresponding untagged versions, the tag is unlikely to alter the motile behaviour. Using gliding assays under multiple motor conditions (motor concentration 0.1–0.5 mg/ml), all investigated constructs were shown to be motile (Table II). Low dilutions of the motors (1:4) already caused a lack of microtubule binding and thus prevented observation of single-molecule gliding events. Within the standard deviation, the NcKin400, 433 and 480 motors were as fast as wild-type full-length NcKin. The monomeric NcKin versions and NcKin391 were also motile, but significantly slower. NcKin391 may be slower than the other dimers because of an unfavourable linkage to the glass surface.

**Steady-state ATPase measurements**

All constructs were also active in basal and microtubule-dependent ATPase assays, with activation factors of ~1300–5700 (Table II). Whereas the dimeric NcKin391, 400, 433 and 480 were essentially indistinguishable from the full-length wild-type motor in the microtubule-dependent ATPase assay with average turnovers of 60–84/s and extremes of 50–104/s, monomeric constructs exhibited distinctly different behaviour. The shortest construct in this study, NcKin343, which lacks the entire neck region, showed an extremely fast ATPase with a turnover of $k_{cat} = 260/s$. The turnover was variable, ranging from 200 to 400/s, probably due to temperature effects. A slightly longer construct, NcKin355, behaved very similarly to the NcKin343 version and yielded $k_{cat}$ values ranging from 75 to 360/s ($n = 5$), with an average of 216/s.

The $k_{cat}$ of NcKin378 was ~10-fold slower than that of NcKin343, with a turnover of $27 ± 10/s$. This low rate is remarkable because all longer constructs, including the full-length wild-type NcKin, exhibit a faster $k_{cat}$ of 60–80/s. However, a slightly shorter (NcKin375) and a slightly longer construct (NcKin383) show similar turnovers ($k_{cat} = 24 ± 6$ and $24 ± 4/s$, respectively). To decide whether these constructs are intrinsically slow, or whether they contain a large number of inactive molecules, the C-terminal end of the neck domain of NcKin378 was digested with a mixture of carboxypeptidase A and B. SDS–PAGE analysis confirmed that the digest yielded one major kinesin fraction that appeared to be intermediate in size between NcKin378 and NcKin343, although the exact extent of the proteolytic degradation is not known. The ATPase activity after the treatment was increased to $50 ± 6/s$ ($n = 2$), corresponding to a 2-fold activation. Similar activation factors were obtained with two carboxypeptidase-treated NcKin375 preparations. The absolute turnover is not as high as in NcKin343 but is clearly above the NcKin378 level, indicating that the partial removal of the neck domain releases an inhibition of catalysis.

These data suggest that dimeric NcKin motors hydrolyse ATP at 60–84/s. The slower rate in NcKin378 and 383 is
probably not due to a large fraction of inactive protein but to an inhibitory action of the neck. NcKin343 without the neck shows a very fast, unrepresed catalysis.

**Chemical processivity**

The steady-state microtubule-stimulated ATPase measurements also contain information on the processive behaviour of the enzyme. In particular, the ratio of the apparent second-order constant $k_{\text{on}}$ATPase = $k_{\text{cat}}/K_{0.5}$MT from steady-state assays, and the measured binding rate, $k_{\text{ub}}$, indicate the number of ATPase cycles induced by one productive diffusional encounter of kinesin and microtubule (Hackney, 1995). This ratio is called the $k_{\text{ub}}$ ratio (‘biochemical processivity’) in Table I. The binding rate $k_{\text{ub}}$ can be determined indirectly by following the ADP release because each productive initial encounter of kinesin with microtubules releases one ADP from the motor. Therefore, NcKin was incubated with a fluorescent ATP species, N-methylnaphthyl-ATP (mBTATP), which yields a kinesin–mBTATP complex because ATP is being hydrolysed quickly, even in the absence of microtubules. The fluorescent decrease upon mixing kinesin with varying, substoichiometric amounts of microtubules was then measured. The use of low microtubule concentrations ensures that the initial binding event is rate limiting, and not the subsequent catalysis which releases the second bound mBTATP of a dimeric kinesin molecule. Hence, kinesin–microtubule binding causes a monoexponential fluorescent decrease, which reflects the rate of the binding reaction. Under microtubule-limiting conditions, this rate depends linearly on the microtubule concentration and reveals the second-order rate constant of kinesin–microtubule binding, $k_{\text{ub}}$ADP.

Table II lists the second-order binding rate constants of the NcKin constructs under investigation, as determined by mBTATP release, $k_{\text{ub}}$ADP. The values were derived from time traces of mBTATP release upon microtubule binding (Figure 2). The binding rates range from $k_{\text{ub}}$ADP = 4.2 to 15.8 μM/s. Most constructs show a $k_{\text{ub}}$ADP at ~4.5 μM/s but the shortest and the longest ones deviate with values of $k_{\text{ub}}$ADP = 15.8 and 13.9 μM/s. These values are comparable with those found for DmKHC (0.6–7.6 μM/s; Jiang et al., 1997) or human kinesin ($V_{\text{max}}/K_{M5} = 40/13 μM = 3.1μM/s; Ma and Taylor, 1995).

The comparison of apparent and measured microtubule binding rate constants ($k_{\text{ub}}$ ratio, Table I) indicates highly divergent chemical processivities for the investigated constructs. The NcKin378 and 383 mutants, comprising the core motor domain, neck-linker and the presumable neck helix, have the lowest values, suggesting that the construct dissociates from the filament after one or a few catalytic cycles. At the other extreme, the NcKin433 construct displays a ratio of 726 and may therefore stay on the microtubule for several hundreds of cycles. The shortest construct, NcKin343, possesses a $k_{\text{ub}}$ ratio of 41 and may stay bound at the microtubule due to the lack of a proper release mechanism (Jiang and Hackney, 1997). These results agree with experiments on truncated DmKHC constructs, with the exception of the monomeric, slow, non-processive NcKin378 and 383 variants whose homologue (DmKHC381) is chemically processive.

![Fig. 2. Second-order rate constant of microtubule-activated mBTATP release, $k_{\text{ub}}$ADP, from NcKin378cys. This example shows the apparent mBTATP release rates upon mixing 100 nM mBTATP–kinesin complex with variable amounts of microtubules (A). The time traces were fit to single exponentials (dotted lines). The resulting apparent rates depend linearly on the microtubule concentration, with the slope of the curve signifying $k_{\text{ub}}$ADP (B). Since the substoichiometric amount of microtubules limits the reaction, the $k_{\text{ub}}$ADP value reflects the rate of kinesin–microtubule binding. The full set of $k_{\text{ub}}$ADP values for all NcKin constructs is listed in Table II.](image_url)

**Discussion**

This study addressed the question of the structural requirements for fast velocity and processive behaviour in conventional kinesins. In animal kinesins, the neck domain appears to confer processive motility to the motors, and its absence leads to mechanically and kinetically aberrant properties (Inoue et al., 1997; Jiang et al., 1997; Young et al., 1998). The findings presented here demonstrate that a fungal kinesin representative, NcKin, shares some features with animal kinesins, but also exhibits several unique and functionally significant properties. Thus, on the one hand, the motile and ATPase characteristics of NcKin clearly depend on the oligomerization state of the molecular motor, as in the animal case. Multiple motor gliding velocities for the shorter, monomeric versions NcKin343 and 378 are significantly slower than for the dimeric constructs, which are indistinguishable from the bacterially expressed full-length kinesin. Similar observations have been made for DmKHC (Young et al., 1998). As in animal kinesins, processivity can only
be observed in dimeric variants. The chemical processivity described here agrees with biophysical studies on NcKin motility (Crevel et al., 1999), and a number of steps similar to processive ATP turnovers reported here have been observed in single-molecule motility assays on a dimeric NcKin483 kinesin (S.Lakämper and E.Meyhöfer, in preparation). Also in agreement with animal kinesins, short NcKin monomers (NcKin343) possess a faster ATPase than dimeric constructs (260 versus 60–80/s). For human kinesin, \( k_{\text{cat}} \) values of 80 and 40/s have been reported for monomeric and dimeric forms, respectively (Ma and Taylor, 1997); for DmKHC, the values are 84 versus 20/s (Gilbert et al., 1995; Moyer et al., 1998) and 64 versus 44/s (Jiang et al., 1997).

On the other hand, there are also obvious differences between fungal and animal kinesins. For one, the fungal NcKin kinesin tail inhibition is less pronounced than in animals, and the dimeric truncated versions NcKin400, 433 and 480 have almost the same \( k_{\text{cat}} \) values as the full-length protein. These findings are in agreement with the observation that the head–tail interaction in NcKin is so salt sensitive that under our assay conditions (ionic strength ~50 mM), the interaction is weakened (Seiler et al., 2000).

The second dissimilarity is the high catalytic rate of the NcKin motor head. The extremely fast ATPase turnover of NcKin343 implies that the catalytic motor core contains determinants for the fast gliding velocity of NcKin. The microtubule-stimulated ATPase rate is >2.5-fold faster than that of the even shorter Drosophila kinesin DmKHC340 \( k_{\text{cat}} = 96/s \), which presumably lacks a part of the functionally important neck-linker and, thus, is expected to display the higher ATPase rate (Jiang et al., 1997; Rice et al., 1999; Tomishige and Vale, 2000). The turnover required to drive motility in steps of 8 nm at 2.6 \( \mu \text{m/s} \) is 165/s per head, assuming one ATP per step. In part, the accelerated ATPase of NcKin compared with DmKHC may be due to a quicker rate of productive binding, as reflected in the apparent bimolecular rate constant, \( k_{\text{b,ADP}} \). For NcKin, it ranges from 4.2 to 15.9/\( \mu \text{M/s} \); DmKHC has values between 0.58 and 7.6/\( \mu \text{M/s} \) (Jiang et al., 1997). It will be interesting in the future to characterize further the striking correlation between the faster NcKin motor core ATPase (~2.5-fold) and the faster gliding velocity of the NcKin motor (~3-fold) compared with animal kinesins in pre-steady-state experiments.

Finally, the most striking difference between fungal and animal kinesins is the dimerization behaviour. Whereas animal kinesins form stable dimers when they contain the neck domain, the fungal NcKin requires additional motifs present in the hinge domain. In animal constructs that dimerize by virtue of their neck domains, dissociation constants below 1 \( \mu \text{M} \) have been reported (Rosenfeld et al., 1996; Jiang et al., 1997). On the basis of NcKin383, a dissociation constant at least 300-fold higher can be estimated. Differences between fungal and animal kinesins become most obvious when comparing NcKin378 and DmKHC381, which both include their respective neck domains and end at homologous residues (Figure 1). Whereas the truncation mutant DmKHC381 is dimeric, the
NcKin378 construct is not. The neck of NcKin thus differs markedly from its animal counterparts.

What causes NcKin to dimerize? As in animal kinesins, the neck domain is able to form an α-helical coiled-coil, as seen in synthetic neck peptides under suitable conditions. Since the neck deletion construct NcKin433-Δneck contains the hinge domain but does not dimerize, the neck coiled-coil most probably causes dimerization. In the context of the protein, however, the neck alone is not sufficient and requires additional elements contributed by the hinge. The mechanism by which the hinge acts on the neck domain remains unclear, but may involve a pKₐ shift of glutamic acid residues, because α-helical coiled-coil formation requires an acidic environment. This pH dependence hints at the importance of protonation of one or more glutamic acid residues in the ε or ε coil positions, as previously observed for coiled-coil model peptides (Kohn et al., 1998). In fact, NcKin contains Glu360 in a potential ε position, which is not found in animal conventional kinesins (Figure 1). Peptides derived from the human kinesin neck were soluble at neutral pH and clearly assumed α-helical coiled-coil conformations (Mori et al., 1997; Tripet et al., 1997).

Dimerization of the NcKin neck in the context of the protein can be induced by the presence of a few key residues from the hinge domain. Thus the NcKin391 mutant was able to dimerize, whereas NcKin383 was not. Apparently, residues 384–391 favour the α-helical coiled-coil conformation of the neck domain. It is unlikely that these residues from the hinge domain contain a trigger sequence as described for other coiled-coils because, in those cases, trigger sequences are part of the α-helical coiled-coil and clearly contain coiled-coil heptad repeats (Burkhard et al., 2000). The hinge, however, has no predicted coiled-coil propensity at all, and contains many residues that are unfavourable for helical structures. Due to the low solubility of expressed hinge peptides, however, we were unable to test its conformational preferences.

Surprisingly, the presence of the neck domain is important for functional characteristics even in the monomeric NcKin378 and 383 versions that probably contain the neck in a non-helical conformation. These constructs displayed the slowest ATPase activities of all mutants. We cannot exclude rigorously that parts of the protein preparations are inactive since the basal activity was also slower by a factor of 2–3, compared with other constructs. However, the activation of NcKin378 by carboxypeptidases demonstrates that the catalytic potential (kcat = 50/s) is at least as high as for fully functional dimeric constructs, suggesting that the ‘free’, undimerized neck domain of the monomer exerts an inhibitory effect on the catalytic core. As it is unknown how far the neck was digested, the inhibition may be even stronger than 1.9-fold [50/s (digested)/27/s (native)].

How does the neck affect ATPase and processivity? One possible hypothesis is that microtubule binding of NcKin378 induces dimerization and thus converts the molecule into a functional dimer (Crevel et al., 1999). However, when mantADP-charged NcKin378 is mixed with microtubules in the absence of ATP, 100% of the fluorescence signal is abolished, arguing for completely
uncoupled heads. In dimeric constructs, only approximately half of the signal is lost (see Supplementary figure 2). The almost complete absence of chemical processivity of NcKin378 agrees with this observation and suggests that the microtubule-activated dimerization of NcKin378 may only be relevant at a high excess of kinesin over tubulin subunits.

Does the neck interact directly with the microtubule, and does this affect the ATPase and its processivity? Lysine residues from the neck have been suspected to interact with the acidic C-term of microtubules and thus to reduce the rate of detachment of the motor from the filament (Thorn et al., 2000; Wang and Sheeet, 2000). In fungal kinesins, three of these lysines are conserved (Figure 1). However, despite the presence of these residues, NcKin378 is not chemically processive.

The neck–microtubule binding model might be extended by hypothesizing an equilibrium between a microtubule-bound form with disordered neck-linker and neck, which hydrolyses ATP quickly, and a slow microtubule-bound form that is tethered to the filament via the neck and an ordered neck-linker (Figure 5A). The conformation with a disordered neck-linker may be captured in the hydrodynamically fast NcKin343 mutant, whereas filament binding of the neck domain in NcKin378 (and longer) induces ordering of the neck-linker and slow ATPase. Fully processive motility could only be established in dimeric constructs due to a strain-dependent release mechanism of the trailing head (Mandelkow and Johnson, 1998).

Apart from being rather speculative, there is one argument against this model. Assuming an additional tether in NcKin378 in comparison with NcKin343, a longer dwell time $\tau$ at the microtubule would be expected. $\tau$ can be estimated by dividing the number of processive hydrolytic cycles ($k_{hi}$ ratio) by $k_{cat}$, yielding $\tau = 41/260 \text{s} = 158 \text{ ms}$ for NcKin343, and $\tau = 6/27 \text{s} = 222 \text{ ms}$ for NcKin378. These values are quite similar, suggesting that either the K–E interactions between neck and filament are weak, or the release of the neck from the microtubule is tightly coupled to the catalytic cycle.

The most straightforward explanation for the observed effects is an inhibition of the catalytic domain by the presence of the neck domain (Figure 5B): NcKin378 and 383 are slower than NcKin343 because elements of the neck (which can be partially removed by C-terminal proteolysis) suppress the ATPase. The inhibition seems to be particularly effective in the absence of a coiled-coil conformation of the neck, and can be relieved by residues of the hinge domain. Protonation of glutamic acid residues in the neck and subsequent coil formation, induced by the presence of the hinge, may be involved in this process by an unknown mechanism. It is conceivable, therefore, that the neck may alternate between a coiled-coil and a molten conformation in the course of the ATP hydrolysis cycle, and this conformational change may be a prerequisite for the fast velocity of fungal kinesins. It will be interesting to test the implications of this model because point mutagenesis might identify single residues responsible for the inhibitory action of NcKin’s neck, and chimeric motor molecules may reveal whether the mechanism of action is a special adaptation of fungal kinesins, or a general feature of conventional kinesins.

Materials and methods

Cloning and expression

Truncated NcKin constructs were cloned using PCR with primers at the 5’ end of the NcKin gene and the desired stop codon. If necessary, a reactive cysteine tag was introduced (PSIVHRKCF*, Funatsu et al, 1997; NcKin480: KLGPVSIVHRKCF*). The resulting fragments were cloned into pT7-based NcKin expression vectors (Hemmesen and Schiwa, 1997), using the internal BstBI site in the NcKin gene, and a PstI site introduced after the stop codon. All plasmids were verified by sequence analysis. For the expression of NcKin protein, media flask with 1 l of TPM (20 g/l tryptone, 15 g/l yeast extract, 2.5 g NaClPO4, 1.0 g NaH2PO4, 10 mM glucose, 100 µg/ml ampicillin) were inoculated from a freshly transformed single colony of Escherichia coli BL21(DE3) (Novagen Corp.), pre-grown for 16–20 h at 22°C, and induced with 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were incubated overnight at 22°C, harvested the next morning and stored at −70°C.

Purification was accomplished as described (Crevel et al., 1999; Supplementary figure 3). Briefly, 4 g of E. coli cells were resuspended in buffer A (20 mM Na- phosphate pH 7.4, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 10 mM dithiothreitol), 10 µM ATP, protease inhibitor mix (Roche Diagnostics), sonifed and centrifuged (35 min at 100,000 g). The supernant was loaded on a 5 ml HiTrap SP Sepharose column (Amersham Pharmacia Biotech) and eluted in a manual NaCl step gradient. The peak fractions were pooled, frozen in liquid N2 with 10% glycerol and stored at −70°C.

C-terminal digestion of protein

To test whether NcKin is inhibited by its neck domain, NcKin378 and 375 preparations were digested using limited carboxypeptidase proteolysis (Ambler, 1972). Carboxypeptidases A and B (Sigma-Aldrich) were added to kinases in 1:10 and 1:20 stoichiometries. The digest was incubated at 22°C and stopped by the addition of 5 mM EDTA after 1 or 2 h.

Oligomerization state

To characterize kinesin’s oligomerization state, sedimentation coefficients $S_{20,w}$ were measured by sucrose density centrifugation, and Stokes radii were determined by gel filtration. $S_{20,w}$ coefficients were determined by centrifugation of 5–10 µM protein solutions on 3–13% sucrose gradient cushions in buffer B (20 mM Na-phosphate pH 7.4, 50 or 200 mM NaCl, 10 µM ATP). The ionic strength was adjusted with NaCl as desired. The rotor (Beckman SW 50.1) was kept at 4°C and run at ~ 15000 rpm (37 000 r.p.m.) for 13 h. Aldolase, bovine serum albumin (BSA) and carbonhydrate (S20,w = 7.4, 4.3 and 3.2, respectively; Roche Diagnostics) were included at 0.1–0.2 mg/ml as internal standards. The gradients were fractionated and analysed on SDS–polyacrylamide gels. The positions of the peaks were used to determine the $S_{20,w}$ density values (NIH Image program, Kaledigraph software).

The proteins’ Stokes radii were determined by gel filtration analysis. Protein solutions (8–35 µM) were loaded on a Superdex 200 column (Amersham Pharmacia Biotech) equilibrated with buffer B containing the desired concentrations of NaCl. The elution volumes of the samples were compared with standard proteins [littorin, 5.9 nm radius; aldolase, 4.5 nm; BSA, 3.55 nm; carbonhydrate, 2.4 nm (or chymotrypsinogen, 2.24 nm), and cytchrome c, 1.64 nm; Roche Diagnostics and Sigma Chemical Corp.]. Stokes radii were calculated from a plot of elution volumes versus standard sizes (Andrews, 1970). The molecular weights of the kinesin constructs were calculated according to the equation of Cantor and Schimmel (1980):

$$M_r = (S_{20,w} \times n_A \times 6 \times \pi \times r_{stokes}^2)/(1 - \phi \times \rho)$$

where $n_A$ is the Avogadro number, $\eta$ is viscosity, $\phi$ is the specific volume of the protein = 0.725 cm³/g and $\rho$ is the density of the medium.

Basal ATPase measurements

The ATPase rates in the absence of microtubules were measured using [γ-32P]ATP (Shimizu et al., 2000). NcKin was incubated in ATPase buffer 12A25 at 3 and 6 mM, along with a blank without kinesin. The reactions were started by the addition of 1 mM [γ-32P]ATP and stopped after 0, 5, 10, 20 and 30 min in 0.3 M perchloric acid in 1 mM NaH2PO4. The reactions were mixed with charcoal to absorb nucleotide and centrifuged to pellet the charcoal. The free [γ-32P]phosphate in the supernatant was quantified in a scintillation counter using Cerenkov radiation. The basal ATPase rates were calculated from linear fits of the time traces.
Steady-state microtubule-ATPase activity

Steady-state ATPase rates were determined using a coupled enzymatic assay (Huang and Hackney, 1994a; Grummt et al., 1998b). The assays were performed in a low ionic strength buffer (ATPase buffer: 12 mM, N-[2-acetamido]-2-aminoethanesulfonic acid (Acet)-KOH pH 6.8, 25 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA). ATP was used at 1 mM; the reactions were started with kinesin. Control experiments omitting single components showed that the kinesin preparations did not contain measurable background activities that might interfere with the coupled assay. Microtubules from pig brain tubulin were polymerized freshly from pre-spin tubulin, stabilized with 20 mM pimeloylactam and centrifuged to remove excess nucleotide. The concentration of the resuspended microtubule solution was determined photometrically at 280 nm (Huang and Hackney, 1994b).

ADP release rates

ADP release rates were measured by mixing mantADP-charged kinesin with stoichiometric amounts of microtubules, and quantification of the fluorescence decrease. To load kinesin with mantADP, the kinesin mutants were incubated with a 4-fold molar excess of mantATP at 25°C for 15 min. The kinesin–mantADP complex was separated from excess nucleotide via Sephadex G25 spin columns. The concentration of the eluted protein was evaluated by a Bradford assay. This protocol was suitable for all truncated NcKin constructs but failed to label full-length NcKin. Kinesin–microtubule binding was monitored in an Amino- Bowman spectrofluorimeter with an excitation wavelength of 365 nm and emission at 445 nm. Kinesin (100–120 nM) was mixed with stoichiometric microtubule concentrations (0–10 nM). The resulting fluorescence decrease was monitored and fitted to single exponential curves from which the release rates were derived. The second mantADP, which is present in dimeric kinesin constructs, is being released in addition of excess ATP at the rate ksub, which is much faster than the rates observed under our experimental conditions. Hence, the mantADP release rates are likely to reflect the rate of productive kinesin–microtubule binding. A plot of the release rates versus microtubule concentrations was used to calculate the bimolecular rate constant, ksub ADP.

Labelling of reactive cysteine tags

NcKin kinesins containing a reactive cysteine tag were labelled with biotin–maleimide. The protein was incubated with a 6-fold molar excess of maleimide conjugate on ice for 60 min. The reaction was stopped with 10 mM DTT. Active kinesin was isolated by a microtubule binding and release step (Vale et al., 1985).

Gilding assays

Motility assays were performed with biotin-labelled kinesins in flow cells coated with streptavidin. After 10 min of incubation with 1 mg/ml streptavidin (in 1x PBS, pH 7.4), the cells were washed with three chamber volumes of blocking buffer (1 mg/ml BSA and 0.2 mg/ml casein in BRB80: 80 mM PIPES–KOH (pH 6.8, 1 mM MgCl2, 1 mM EGTA), and filled with biotin-labelled kinesin in blocking buffer. The assays were started after 10 min by flowing the chambers with blocking buffer containing 6 mM MgCl2, 5 mM ATP, 5 mM phosphoenolpyruvate, 200 mM KCl, 20 mM pimeloylactam and microtubules. Motility was monitored in a Zeiss Axioshot using video-enhanced phase-contrast microscopy.

Peptide synthesis and purification

Two peptides derived from the NcKin neck region were used in this study, termed Kn1 and Kn3. The latter is a peptide dimer cross-linked by a disulfide bond between cysteine residues located in the first position of the heptad repeat of the presumed coiled-coil and was chosen to match the optimal location of disulfide bonds in supercoils according to previous studies (Figure 1; Kohn et al., 1998). Whereas peptide Kn1 was synthesized automatically by solid-phase 9-fluorenylmethoxycarbonyl- (Fmoc)/tBu chemistry on TentaGel S-RAM resin on an Applied Biosystems model 431A peptide synthesizer, peptide Kn3 was synthesized manually with standard Boc/Bzl chemistry on a 4-methylbenzhydrylamine resin. For details, see Supplementary data.

Circular dichroism

The CD spectra were recorded on a Jasco J-715 spectropolarimeter at 20°C in quartz cuvettes (temperature controller PFD-350S). The concentrations of the filtered samples (0.45 μm) were determined by absorbance at 280 nm. The spectra (average of 10 scans) were normalized to their mean residue molar ellipticity [Θ]250 (°cm²/dmol). The spectra were recorded between 180 and 250 nm, with a scanning speed of 50 nm/min, a response of 1 s and a band width of 1.0 nm. Melting curves were measured by following the change of molar ellipticity at 222 nm versus temperature, with a temperature slope of 30°C/min, a response of 1 s and a band width of 1 nm. The melting temperature (Tm) was derived from the zero intercept of the second derivative of the respective melting curve.

Mass spectrometry

The mass spectra were taken on a PE SCIXE API 165 single quadrupole MS system. The spectrum for detecting the non-covalently bound dimers was collected in the range 700–2000 Da, with an infusion pump rate of 0.3 μl/min, an ion source high voltage of 4.5 kV, an orifice voltage of 15 V, a dwell time of 0.6 ns per scan and a step size of 0.2 Da with 10 scans summed.

Supplementary data

Supplementary data for this paper are available at The EMBO Journal Online.

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