Importance of a flexible hinge near the motor domain in kinesin-driven motility

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Conventional kinesin is a molecular motor consisting of an N-terminal catalytic motor domain, an extended stalk and a small globular C-terminus. Whereas the structure and function of the catalytic motor domain has been investigated, little is known about the function of domains outside the globular head. A short coiled-coil region adjacent to the motor domain, termed the neck, is known to be important for dimerization and may be required for kinesin processivity. We now provide evidence that a helix-disrupting hinge region (hinge 1) that separates the neck from the first extended coiled-coil of the stalk plays an essential role in basic motor activity. A fast fungal kinesin from *Syncephalastrum racemosum* was used for these studies. Deletion, substitution by a coiled-coil and truncation of the hinge 1 region all reduce motor speed and uncouple ATP turnover from gliding velocity. Insertion of hinge 1 regions from two conventional kinesins, Nkin and DmKHC, fully restores motor activity, whereas insertion of putative flexible linkers of other proteins does not, suggesting that hinge 1 regions of conventional kinesins can functionally replace each other. We suggest that this region is essential for kinesin movement in its promotion of chemo-mechanical coupling of the two heads and therefore the functional motor domain should be redefined to include not only the catalytic head but also the adjacent neck and hinge 1 domains.
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Introduction

Conventional kinesin is a microtubule-based motor enzyme that uses the energy derived from ATP hydrolysis to move unidirectionally along microtubules (Bloom and Endow, 1994; Hirokawa, 1998). Native, functional kinesin consists of two heavy chains with N-terminal motor domains followed by an extended coiled-coil stalk and a C-terminal globular tail domain (Figure 1). In addition, animal conventional kinesins have two light chains associated with the tail (Hirokawa *et al.*, 1989; Yang *et al.*, 1989). The catalytic motor domain contains the ATP-binding site and the microtubule-binding site, and its three-dimensional structure is known (Kull *et al.*, 1996; Sablin *et al.*, 1996). The catalytic domain is followed by

the neck region, which in turn can be divided into the neck linker, characterized by two short β sheets (Kozielski *et al.*, 1997), and the neck coiled-coil (Morii *et al.*, 1997; Tripet *et al.*, 1997) thought to be responsible for dimerization (Huang *et al.*, 1994; Berliner *et al.*, 1995; Young *et al.*, 1995) and chemo-mechanical coupling (Hackney, 1994; Jiang *et al.*, 1997). The neck is followed by a segment of variable length with poor sequence conservation that is predicted not to form a coiled-coil. In fungal kinesins, this region, referred to as hinge 1, contains several proline and glycine residues which signify flexibility. The bulk of the stalk that follows is α -helical in nature and apparently forms an extended coiled-coil. It is interrupted by a short, flexible segment, termed kink or hinge 2, whose existence has been linked to the ability of the dimer to fold back on itself (Hirokawa *et al.*, 1989; Hackney *et al.*, 1992).

Little is known about the possible contribution of elements of the molecule outside the head/neck region to the motility properties of kinesin. To address this question, we have made a number of deletion and insertion constructs in domains of the kinesin molecule outside the catalytic motor domain using kinesin of the zygomycete fungus *Syncephalastrum racemosum* as a model. Just as with conventional kinesin of *Neurospora crassa* (Steinberg and Schliwa, 1995), the conventional kinesin of *Syncephalastrum* (which we have termed Synkin) is a homodimer that moves microtubules in gliding assays at 2.1–3.4 $\mu\text{m/s}$, i.e. 3- to 5-fold faster than its animal counterparts (Steinberg, 1997). Synkin has been cloned and expressed in bacteria, and the bacterially expressed full-length molecule has been shown to be a homodimer that moves microtubules in gliding assays at the same speed as native Synkin isolated from *Syncephalastrum* cell extracts (Grummt *et al.*, 1998). Therefore, Synkin promises to be a sensitive model system for studying the motile properties of conventional kinesins.

Here, we present evidence that the presumably flexible domain that immediately follows the neck coiled-coil, termed hinge 1, is essential for optimal motility of dimeric kinesin. The constructs produced and analysed in this study were made in such a way that the overall structural organization of the motor remained undisturbed; i.e. the constructs still had physical properties consistent with a dimeric, extended molecule. We show here that alteration of the hinge 1 domain dramatically affects kinesin motility, and that hinge 1 domains of different kinesins can functionally replace one another. These findings extend the functional motor domain to include not only the catalytic head, but also a considerable portion of the stalk up to the first extended coiled-coil.

Results

Sequence comparisons of fungal kinesins revealed the presence of a region 40–50 amino acids in length

Fig. 1. Schematic overview of the domain organization of *Syncephastrum* kinesin (Synkin) showing the positions of the primers used for the construction of the deletion mutants (for details see text). Dark grey: motor domain. Light grey: regions predicted to form a coiled-coil. N, neck; H, hinge 1; C1, coil 1; K, kink or hinge 2; C2, coil 2.

Fig. 2. Sequence organization of the neck and hinge 1 regions of the conventional kinesins from *Syncephastrum* (Synkin), *Neurospora* (Nkin) and *Drosophila* (DmKHC). The approximate positions of the neck and hinge 1 domains are indicated on the bottom. The coiled-coil prediction of the Synkin neck is shorter by one heptad. Conserved peptide motifs flanking the hinge 1 region are shaded in light grey. The regions deleted in the constructs ΔpH and ΔH are indicated at the top.

C-terminal to the neck domain that is relatively rich in prolines (Figure 2). Even though there is little sequence similarity in this region among the fungal kinesins, it probably constitutes a functional domain because of the presence of highly conserved amino acid residues flanking this region on both sides [WRXGXXV at the N-terminal side and D-E/D-R/K-E/D-E/D at the C-terminal end]. Since these conserved flanking amino acid residues are also present in animal kinesins, the functional homology of the enclosed domain can be extended to all conventional kinesins. In both fungal and animal kinesins this domain, which has been termed linker or hinge 1 (Howard, 1996; Vale and Fletterick, 1997), is not predicted to form a coiled-coil and therefore separates the coiled-coils of the neck and the first extended coiled-coil (coil 1) of the stalk (see Figure 2).

To determine the potential role of hinge 1 in motor mechanics and motility, we have generated a series of deletion and insertion constructs.

Deletion mutants

Constructs with deletions in different regions of the stalk were generated, expressed in *Escherichia coli* and tested for motility in gliding assays. The results are summarized in Figure 3. The average velocities of the construct ΔdC1K, which lacks the distal half of coil 1 plus the kink, and the construct ΔT, in which an interruption of coil 2 was deleted, were almost identical to that of wild-type Synkin (2.54 and 2.57 μm/s, respectively). The average velocity of ΔC1K, in which the entire first, long coiled-coil segment plus the kink region were deleted, was also only slightly slower than wild-type Synkin (2.05 μm/s). These results indicate that regions C-terminal to the linker are not essential for the motility of Synkin. In contrast, deletion of either the neck (ΔN) or the hinge 1 region (ΔH) of Synkin leads to a dramatic reduction in the velocity of microtubule gliding (Figure 3). The importance of these regions is further emphasized by the behaviour of the constructs ΔNH and ΔNHC1, in which both regions, alone

Fig. 3. Structure and velocity of microtubule gliding of the deletion constructs. The motor domain is shown in dark grey, and coiled-coil regions of the stalk in light grey. Velocities are shown as mean ± SD from 3–10 independent experiments, each with at least five microtubules. Microtubule gliding activity of the construct ΔNHC1 was measured in S2 rather than S5. N, neck; H, hinge 1; C1, coil 1; K, kink or hinge 2; C2, coil 2.

or in combination with coil 1, have been deleted. These constructs show that the neck and the hinge 1 region are important for the motility properties of Synkin. Interestingly, the construct ΔpH, in which just the proximal portion of the hinge 1 region is missing, also moved with a dramatically decreased velocity. This suggests that even a shortened hinge 1 is insufficient to support optimal performance of the motor molecule.

Insertion mutants

The observation that deletion of all or part of the hinge 1 region severely hampers motor performance is unexpected,

since this segment of the stalk is separated from the catalytic core domain by the neck. This prompted us to investigate the structural requirements of this region by replacing it with other sequences. The results are summarized in Figure 4. First we addressed the question of whether other sequences with a similar content and distribution of proline residues can replace the Synkin hinge 1. Therefore, proline-containing sequences from two actin-binding proteins of *Dictyostelium* (CAP50 and protovillin) were inserted instead of the proline-rich region of Synkin (Figure 5). These two sequences contained a similar number of proline residues as the Synkin linker; both are predicted to act as hinges between globular domains in both CAP50 (Gottwald *et al.*, 1996) and protovillin (Hofmann *et al.*, 1993). The CAP50 and protovillin constructs expressed in bacteria were largely insoluble, therefore motility was not observed in bacterial extracts (S2). However, in both cases a functional motor could be enriched by a microtubule affinity step. The purified CAP50 mutant supported slow microtubule gliding (0.33 $\mu\text{m/s}$). In preparations of the purified protovillin mutant, poor microtubule attachment to the coverslip was observed. The behaviour of both constructs indicates that domains with a relatively high proline content that act as flexible hinges in other proteins are unable to execute the same function as the authentic hinge 1 region.

In a second type of insertion experiment, hinge 1 was replaced by a segment of coiled-coil taken from the middle of coil 2. Care was taken to execute this insertion in such a way that the coiled-coil characteristics were not disturbed. Thus, an extended, uninterrupted domain with an optimal heptad repeat pattern was created that included the neck, the insert and coil 1. With this construct the speed of microtubule gliding varied from preparation to

Fig. 4. Structure and velocity of microtubule gliding of the insertion constructs. CAP50, linker region of *Dictyostelium* CAP50. coil, insertion of the segment V657–Q692 from coil 2 of Synkin to create a continuous coiled-coil that includes the neck, insert and coil 1. The velocity shown is that of the preparation with the highest velocity. Other independent preparations showed a slower average velocity or microtubule attachment only. NcH, insertion of the Nkin hinge 1. DmH, insertion of the DmKHC hinge 1.

preparation. In the best preparation it was $0.56 \pm 0.16 \mu\text{m/s}$, but it was considerably slower in others.

We then replaced the hinge 1 region of Synkin with that of another conventional kinesin, the *Neurospora* motor Nkin, to determine whether these domains can functionally replace each other (Figure 5). This construct (NcH) was soluble and moved microtubules in gliding assays almost as fast as wild-type Synkin (average = $2.52 \mu\text{m/s}$). We also inserted the corresponding hinge 1 domain from *Drosophila* kinesin heavy chain (Figure 5), which contains only one proline residue. The resulting construct, DmH, moves microtubules with $2.33 \mu\text{m/s}$, almost as fast as wild-type Synkin. This demonstrates that hinge 1 regions of different kinesins can functionally replace one other, suggesting that these regions of kinesins share certain structural or biochemical characteristics. This finding is surprising, since kinesins do not have any sequence similarity in the hinge 1-domain and may vary considerably in length. The behaviour of the construct with the *Drosophila* hinge 1 (DmH) also showed that a relatively high proline content, which is characteristic of the fast fungal kinesins, is not a prerequisite for a high motor velocity.

Oligomerization state of the constructs

To determine whether the constructs were in fact dimers and behaved similarly to wild-type Synkin, the hydrodynamic behaviour of several of the constructs has been determined (Figure 6). In all cases, pairs of constructs were loaded on either the gel filtration column or the sucrose density gradient in order to pick up subtle differences in their behaviour. Two examples of these pairwise comparisons are shown in Figure 6, and the results of all the constructs that were studied are summarized in Table I. The hydrodynamic behaviour of ΔdC1K (Stoke's radius, 6.9 nm; Sw20, 8.0S; and mol. wt_{calc} 232 kDa) and ΔN (6.9 nm/8.3S/236 kDa) is almost identical to that of wild-type Synkin (7.0 nm/8.0S/232 kDa). For the constructs ΔH and ΔpH , both the Stoke's radius (7.5 and 7.3 nm, respectively) and the Sw20 (9.1 and 8.7S, respectively) were slightly increased, resulting in a higher calculated

Fig. 6. Behaviour of Synkin and two of the deletion mutants in both gel filtration chromatography and sucrose density gradients using the method of pairwise comparison. For a summary of the results, see Table I.

Synkin	GGTVP –EGKWVTMDKVS KGDFAGLPPAPGFKSPVSDEG ----- SRPATP
CAP50	GGTVPR GGDAKSATPAPASSAPAAPVAVSSTPVE----- SRPATP
PROTOVILLIN	GGTVPR TTAATPKPITTPVTTPPKPITTPVATLKTVT----- SRPATP
Nkin	GGTVPRE KWVPPLELAITPSKSASTARPTPSRLLPESRAETPAISD----- SRPATP
DmH	GGTVPRE EQINMEDLM EASTPNLEVEAAQTAAEAALAQR TALANMSASVAVNEQ SRPATP

Fig. 5. Comparison of the sequence of the Synkin hinge 1 with the sequences inserted in the insertion mutants. For all constructs the flanking Synkin sequences are shown in bold.

mol. wt (283 and 262 kDa, respectively). Apparently, deletions in the hinge 1 region influence the shapes of these molecules. Nevertheless, for these and the other constructs, the data are consistent with the existence of dimers rather than monomers. Mixed oligomeric states can be excluded on the grounds of the sharp focus in the fractionation experiments (Figure 6), and a trimeric state seems highly unlikely.

ATPase activity

To determine whether the altered motility behaviour of the hinge 1 mutations was due to enzymatic defects, ATPase assays were performed. For these assays, C-terminally truncated constructs (amino acids 1–612) were used to prevent kinetic inhibition by the tail (Hackney, 1996). Also, a His-tag was introduced to facilitate purification. Table II correlates ATP turnover, gliding velocity and the Michaelis–Menten constant K_m (MT) of the deletion and insertion constructs. Whereas wild-type Synkin had a microtubule-stimulated ATPase activity with a turnover of 32 ± 5.9 ATP/head/s, mutants containing deletions in their neck and/or hinge 1 regions exhibited a much higher ATP turnover. Replacement of the hinge 1 region with the homologous domains of either *Drosophila* or *Neurospora* to the hinge 1 region of CAP50 restored ATP turnover to that of wild-type Synkin. For the fast constructs there is a discrepancy between gliding velocity and ATP turnover, the latter being too low to explain the rates of movement observed. Similar observations have also been made by others using constructs other than the catalytic motor domain alone (e.g. Sadhu and Taylor, 1992; Gilbert and Johnson, 1993; Berliner *et al.*, 1994). Nevertheless, since the deletion mutant ATPases show a gain-of-function phenotype, the comparison with wild-type Synkin probably reflects altered enzymatic behaviour.

Large differences were seen in the K_m for microtubules. In the deletion mutants, the K_m (MT) was increased ~2- to 6-fold. In the hinge replacement mutants *DmH* and *CAP50*, the values for K_m (MT) were increased 50- and 15-fold, respectively, whereas the value for *NcH* was even slightly lower than that of the wild-type construct. Why the K_m (MT) varies so strongly in the three insertions constructs while the k_{cat} is restored to wild-type levels remains to be determined. Kinetically, the hinge replacement mutant *NcH* resembles wild-type Synkin most closely, in agreement with the higher degree of sequence similarity between these two fungal kinesins. Relating ATP turnover to gliding velocity (coupling factor; Table II) clearly demonstrated uncoupling of these two parameters in the deletion mutants and complete restoration of coupling in the hinge replacement mutants *DH* and *NcH*.

Table I. Hydrodynamic behaviour of different Synkin constructs

Construct	Stoke's radius	Sw20	mol. wt	<i>n</i>	
Synkin	7.0 ± 0.2	8.0 ± 1.2	232 ± 46	6	dimer
ΔdC1K	6.9 ± 0.1	8.1 ± 0.4	233 ± 20	4	dimer
ΔN	6.9 ± 0.1	8.3 ± 0.4	236 ± 8	3	dimer
ΔH	7.5 ± 0.1	9.1	283	3	dimer ^a
ΔpH	7.3	8.7	262	1	dimer ^a

^aSee text for discussion.

Discussion

Recent work has identified the region of the stalk immediately adjacent to the conserved motor domain, the neck, as an element important for motor function and polarity of movement (Case *et al.*, 1997; Cross, 1997; Henningsen and Schliwa, 1997; Endow and Fletterick, 1998; Romberg *et al.*, 1998). Here we show that a presumably flexible hinge region immediately C-terminal to the neck is of crucial importance for motor activity. This domain separates the neck from the first extended coiled-coil of the stalk. If it is deleted, truncated or altered, motor velocity is reduced 4- to 5-fold. Insertion of positionally homologous domains from other kinesins fully restores motor function. This finding is surprising and unexpected: surprising because this domain is located at some distance from the conserved catalytic core domain, and unexpected because of a lack of sequence similarity in this domain, even in closely related kinesins. Thus, not only the catalytic motor domain itself, but also regions of the molecule previously believed to be part of the stalk, are important for motor mechanics.

Since bacterial expression of Synkin resulted in a fully functional motor, it was possible to study the role of different domains of this molecule in deletion experiments. In contrast to previous truncation experiments (Stewart *et al.*, 1993; Berliner *et al.*, 1995; Young *et al.*, 1995; Vale *et al.*, 1996; Inoue *et al.*, 1997), we created internal deletions that yielded a functional motor in an overall nearly native configuration. This approach was chosen because mechanistically dimeric motors differ markedly from single motor domain constructs, even though certain single-headed constructs are capable of generating microtubule movement of high velocity (Inoue *et al.*, 1997). Deletions behind the hinge 1 region, e.g. ΔpC1K, ΔT and ΔC1K, resulted in recombinant protein which supported microtubule gliding with velocities comparable to wild-type Synkin. These observations are in agreement with C-terminal truncation experiments in animal kinesins, in which all dimeric constructs that still possessed the neck and hinge 1 (up to amino acids position ~440) moved microtubules with near wild-type velocity (Stewart *et al.*, 1993; Berliner *et al.*, 1995; Young *et al.*, 1995). So these findings, and ours, show that domains of the stalk C-terminal to the beginning of coil 1 are not directly involved in the motor activity of dimeric constructs.

In contrast, manipulations that affect the neck and hinge domains next to the catalytic domain markedly affect motor function. The neck coiled-coil is highly conserved among animal conventional kinesins (~60% amino acid

Table II. Enzymatic characterization of mutant constructs

Construct	K_m (MT) (nM tubulin)	k_{cat} (ATP/head/s)	Gliding velocity (μm/s)	Coupling rate ($k_{cat}/gliding$)
Synkin-His	86 ± 19	32 ± 5.9	2.62	1
ΔN-His	334 ± 21	153 ± 4.2	0.87	14
ΔH-His	543 ± 6	81 ± 14	0.68	10
ΔNH-His	192 ± 10	84 ± 5.4	0.53	13
<i>DmH</i> -His	4403 ± 335	38 ± 5.4	2.33	1.3
<i>NcH</i> -His	25 ± 11	23 ± 1.4	2.52	0.8
<i>CAP50</i> -His	1311 ± 159	34 ± 14	0.33	8

identity) and similarly highly conserved among fungal conventional kinesins, but only relatively poorly conserved between these two subfamilies (~25% identity). Based on enzymatic and structural studies, it may play a role in interdimer communication (Hackney, 1994; Arnal *et al.*, 1996; Hirose *et al.*, 1996; Tripet *et al.*, 1997). The importance of the neck domain is further supported by the deletion experiment reported here for a fungal kinesin, as well as by a number of substitution experiments in human kinesin (Romberg *et al.*, 1998). The deletion of Synkin's neck (ΔN) results in a construct with clearly decreased velocity (about one-third of wild-type Synkin). In contrast, duplication, stabilization or other modifications of the neck in human kinesin decrease motor velocity only slightly (Romberg *et al.*, 1998). Surprisingly, these alterations reduce but do not abolish processivity, as measured in a single-molecule assay (Romberg *et al.*, 1998). Thus, in human kinesin the neck enhances motility and processivity, but its modification does not appear to abolish basic motor functions.

Immediately C-terminal of the neck lies a stretch of 40–50 amino acids that we show here to be a functionally important domain. This hinge 1 region is characterized by rather inconspicuous sequence attributes: a low probability for structural features such as α -helix, β -sheet or coiled-coil, and, in fungal conventional kinesins, a relatively high proline content (~25%). Other noteworthy features are the striking lack of sequence identity in this domain and the presence of short flanking sequence motifs that are highly conserved between fungal and animal kinesins: the motif WRXGXXV on the N-terminal side and a cluster of mostly negatively charged amino acids on the C-terminal side.

The strongest support for the hinge 1 region being a distinct functional domain comes from the deletion and insertion experiments which were motivated initially by the conspicuous proline content of this region in fungal kinesins. Its partial or complete deletion leads to a dramatic decrease in motor velocity. An important role in motor function was confirmed by insertion of the homologous domain of Nkin, which fully restores Synkin motility. Unexpectedly, however, so does the corresponding region of *Drosophila* kinesin, even though it contains only one proline residue. That it is not the number and distribution of proline residues that is important is confirmed by the insertion of the CAP50 linker, which has a similar number and overall distribution of proline residues as the Synkin hinge 1, yet does not restore motility. This suggests that the hinge 1 regions of conventional kinesins have properties that cannot easily be replaced. The sequence features that confer these properties remain obscure at present. Thus, in the hinge 1 regions of conventional kinesins known to-date, the proportion of non-polar amino acids varies between 35 and 60%, and the distribution of charged or polar amino acids does not reveal any obvious pattern and may diverge considerably. Nevertheless, despite the lack of unifying sequence features, the functional studies reported here assign the hinge 1 domain an important role in kinesin movement.

What is the possible role of this domain in kinesin motility? It is probably a flexible region: poor sequence conservation, the low propensity for α -helix and β -sheet formation, the proline/glycine content and the suscepti-

bility to proteolysis (Ingold *et al.*, 1988; Kuznetsov *et al.*, 1989) all are consistent with this view. Although hinge 1 is not essential for motility *per se*, it does make kinesin movement highly efficient. The velocity of microtubule gliding is at least four times faster when this hinge is present and five to eight times faster than in constructs with linker sequences of other proteins (CAP50, protovillin). Furthermore, these alterations uncouple gliding velocity and enzymatic activity.

In the rotary engine model suggested by Howard (1996), the hinge 1 region would help to relieve torsion generated by the movement of the dimeric motor. Some of the torsion could be stored in such a flexible domain and would be finally relieved by rotation of the kinesin receptor in the fluid membrane of the organelle. Alternatively, the problem of permanent rotation of the motor molecule could be circumvented if the two motor domains alternate between clockwise and counterclockwise turning to bring the trailing head forward. A flexible connection between the two motor units and the extended stalk domain would support this mode of movement. However, this model implies that kinesin takes non-equivalent steps, which is in contradiction to the rotational symmetry of the dimeric motor. Walking or limping models (Block and Svoboda, 1995; Cross, 1995; Hirose *et al.*, 1996; Kozielski *et al.*, 1997; Thormählen *et al.*, 1998) assume that kinesin moves towards the next binding site on the microtubule by a 'step'. Such a step is probably accompanied by a substantial movement of the neck, as suggested by the results of Hirose *et al.* (1995). A flexible linker at the end of the neck could support these movements without disturbing the orientation of the tail domain relative to the cargo.

As an alternative model, the hinge 1 region might enhance chemo-mechanical coupling of the two heads, as indicated by the apparent uncoupling of ATPase activity and gliding velocity in mutant constructs. Full structural integrity of the neck and the linker might be required to develop the physiological forward bias of the motor. It is currently not known how kinesin directs rebinding of the unbound head to a 'forward' binding site on the microtubule. The hinge 1 region may contribute to orient the unbound motor domain properly in the potential field that exists above the microtubule surface. Mutations and deletions in the hinge 1 region may shift the dimer structure towards conformations that position the unbound head in a less favourable manner and make backwards steps more likely. This would explain both the existence of the conserved flanking amino acids and the observation that hinge 1 of Synkin can be functionally replaced by homologous hinges of kinesins, but not by other sequences that are believed to act as flexible linkers. Thus the function of hinge 1 would be more subtle than simply serving as a flexible element: it would enhance the coupling of the chemical cycle of ATP hydrolysis and the mechanical cycle of binding and release of the motor head. Since k_{cat} and K_m (MT) are linked to chemical processivity of kinesin (Hackney, 1995), the mutant ATPase activities indicate an influence of the hinge 1 region on processivity. It remains to be determined whether Synkin and the constructs studied here are processive; nevertheless, the multiple motor gliding assay has revealed that even basic motor performance is severely affected by alterations in the hinge 1 region.

The findings reported here provide evidence for the importance of the linkage between the catalytic head and the extended coil-1-region of the stalk for optimizing kinesin movement and for proper kinetic behaviour. Whereas the catalytic motor domain (i.e. the first ~340 amino acids) is essential for ATP hydrolysis and the initiation of a conformational change, the functional motor domain includes not only the neck but also the hinge 1 region, since it seems to be required for efficient motility. Further work is needed to uncover the design principles of this important domain of the kinesin molecule.

Materials and methods

Materials

Unless otherwise noted, reagents and enzymes were purchased from Sigma (Deisenhofen) or Merck (Darmstadt), and restriction enzymes and modifying enzymes from New England Biolabs (Schwalbach) or Eurogentec (Seraing, Belgium).

Construction of the deletion mutants

Deletion constructs of Synkin were created by amplifying the vector Synkin-pT7 (Grummt *et al.*, 1998) with primers carrying an *Afl*III restriction site at their 5' end. The positions of the primers are shown in Figure 1. The primers pointed outward and were named according to the amino-acid sequence at the corresponding position in the protein. The PCR products were digested with *Afl*III, ligated and transformed into *E. coli* strain BL21. If the bacteria expressed protein of the expected length, the deletion vector was purified and sequenced in the region between a *Bsm*I site and a *Pml*I site (bp 974 and 1960 in the coding sequence of the Synkin cDNA). When the sequence of this region was correct, the *Bsm*I–*Pml*I fragment was isolated and cloned in the Synkin-pT7 vector, which was cut with the same enzymes.

The different deletion mutants were amplified with the following primer combinations: Δ C1K, VDSL⁵⁰⁶ + KMLV⁶⁰¹; Δ N, NADL³³⁹ + KWVT³⁸⁵; Δ H, IAAL³⁶⁵ + KREN⁴³⁴, Δ pH, IAAL³⁶⁵ + KSPV⁴⁰⁷; Δ T, QAAL⁷¹⁷ + KDIE⁷³⁹; Δ NH, NADL³³⁹ + KREN⁴³⁴; Δ NHC1, NADL³³⁹ + KMAQ⁵⁴⁹ and Δ C1K, VPTL⁴²¹ + KMLV⁶⁰¹.

Construction of the insertion mutants

The vector for the insertion mutants was created by inverted PCR with the primers GGTV–*Sac*II (5'-TCG CCG CGG TAC TGT ACC GCC TGT-3') and *Nru*I–PATP (5'-GGG TCG CGA CCG GCT ACG CCC GTG CC-3'). The fragment was blunt, phosphorylated (SureClone kit, Pharmacia) and ligated. This vector was amplified in *E. coli*, digested with *Sac*II and *Nru*I, and treated with calf intestine phosphatase. The inserts of the different constructs were created with the following primer combinations: CAP50: *Sac*II–GGDA (5'-GCA CCG CGG GGT GGA GAT GCT AAA TC-3') and TPVE–*Nru*I (5'-AAG GGT CGC GAT TCA ACT GGA GTG GAA-3'), Protovillin: *Sac*II–TTAA (5'-GCA CCG CGG ACA ACA GCA GCT ACT CC-3') and KTVT–*Nru*I (5'-AAG GGT CGC GAT GAT GAT GGT TTT AGA G-3'), *DmH*: *Sac*II–EEQI (5'-TCC CCG CGG GAG GAG CAA ATC AAC ATG-3') and VNEQ–*Nru*I (5'-ACC ATC GCG ACT GCT CGT TCA CGG CAA C-3'), *NcH*: *Sac*II–EKVV (5'-TCC CCG CGG GAG AAA TGG GTA CCA CCT-3') and AISD–*Nru*I (5'-ACC ATC GCG AGT CTG AGA TAG CAG GGG-3'). The inserts were digested with *Sac*II and *Nru*I and ligated with the insertion vector. The mutant 'coil' in which part of coil 2 (amino acids 657–692) was inserted instead of the linker region was created by using a vector generated by inverted PCR with the primers WRTG–*Kpn*I (5'-GGG GTA CCG CCT GTA CGC CA-3') and *Nru*I–TLEK (5'-AAG GGT CGC GAA CTC TCG AGA AGG ACG A-3'). The insert was amplified using the primers *Kpn*I–VDNL (5'-GGG GTA CCG TAG ACA ACC TCT CTG-3') and DRKQ–*Nru*I (5'-AAG GGT CGC GAT TGC TTG CGA TCC AA-3'). Both PCR products were ligated together after *Kpn*I–*Nru*I digestion.

The constructs were then checked for expression of the protein and the correct sequence as described for the deletion constructs.

Construction of the His-tag mutants

A 180 bp fragment of the vector pQE16 containing the his-tag and the termination region was amplified with the primers 5'-TGA TAT CCC GGG CAT CAC CAT CAC CAT CAC-3' and 5'-CG GGA TCC TTA

GCT CCT GAA AAT C-3'. This fragment was cloned in pUC18 (SureClone kit, Pharmacia). The 180 bp fragment was isolated by digesting the resulting vector with *Eco*RV and *Bam*HI. The vector Synkin-pT7 and the mutated vectors were digested with *Pvu*II and *Bam*HI, treated with calf intestine phosphatase and ligated with the 180 bp fragment. The resulting constructs end with the sequence: SDLS⁶¹²DPGHHHHHH.

Preparation of microtubules

Tubulin was purified from pig brain using three cycles of polymerization and depolymerization according to the method of Shelanski *et al.* (1973) with modifications described previously (Mandelkow *et al.*, 1985). Microtubules were polymerized at a concentration of 1–15 mg/ml in the presence of 1 mM GTP and 10% dimethyl sulfoxide (DMSO).

Bacterial expression and purification of the motor constructs

The vector Synkin-pT7 was transformed into *E. coli* strain BL21. Five hundred millilitres of bacterial culture ($OD_{600} = 0.6$) were induced overnight with 0.2 mM IPTG at 20°C. The bacteria were harvested (Sorvall GSA rotor, 6000 r.p.m. for 10 min at 4°C) and resuspended in 12 ml of AP100 (100 mM PIPES, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA) containing a cocktail of protease inhibitors (100 μ M Pefabloc SC, 10 μ g/ml tosyl-arginine-methyl ester, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml pepstatin, 2.5 μ g/ml leupeptin), 1 mM dithiothreitol and 10 μ M ATP. After sonication (4 $\times$ 30 s, output 4, Branson Sonifier 250) the lysed cells were centrifuged (Beckman rotor 42.1, 42 000 r.p.m. for 60 min at 4°C) and the supernatant (S2) was collected. After addition of taxol-stabilized microtubules (0.3–0.5 mg/ml S2), 7 μ M taxol, 200 μ M AMP–PNP and apyrase (5 U/ml S2), the mixture was incubated on ice for 15–60 min. The microtubules were sedimented at 100 000 *g* for 30 min and centrifuged through a 10% sucrose cushion. The resulting microtubule pellet was resuspended in AP100 in the presence of 5–10 mM ATP and placed on ice for 10 min. After centrifugation at 100 000 *g* for 30 min, the resulting supernatant (S5) in which the only major contaminant was tubulin (Grummt *et al.*, 1998) was tested in a gliding assay.

His-tag constructs were purified from bacterial S2 (see above) using Ni-NTA agarose. One millilitre of the washed resin was incubated with S2 for 1 h at 4°C on a roller. The mixture was transferred to a column, rinsed first with wash buffer (50 mM phosphate buffer pH 6, 100 μ M ATP, 250 mM NaCl, 1 mM MgCl₂), followed by wash buffer plus 60 mM imidazole, and eluted with wash buffer plus 250 mM imidazole. Peak fractions were pooled and protein concentrations were determined on Coomassie-stained SDS gels with bovine serum albumin as standard. The protein concentration was between 0.1 and 0.6 mg/ml. The motility of the peak fractions (diluted 1:10) was tested in gliding assays (see below).

Motility assay

For microtubule gliding assays, 4 μ l of S5 were placed on a coverslip for 1–3 min and taxol-stabilized microtubules and 4 mM ATP were added. The coverslip was sealed with VALAP (vaseline, lanolin, paraffin at 1:1:1), and motility was monitored by video-enhanced light microscopy using a Zeiss axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Hamamatsu DVS 1000 Image Processing system (Hamamatsu Photonics, Herrsching, Germany). Images were recorded on a Panasonic AG 6720 video recorder.

ATPase assay

To characterize the MT-dependent ATPase activity, a coupled enzymatic assay was used (Huang and Hackney, 1994). In a final volume of 80 μ l ATPase buffer (25 mM K-acetate, 12 mM ACES–KOH pH 6.8, 2 mM Mg-acetate, 0.5 mM EGTA) containing 0.5 mM MgATP, 5 mM phospho(enol)pyruvate, 0.2 mM NADH, 2 U lactate dehydrogenase, 5 U pyruvate kinase, 0–20 μ M microtubules were added. The ATPase reaction was started by adding 50–100 nM kinesin to the assay, and $\Delta E_{340}/\text{min}$ was used to calculate activities. Tubulin supplemented with 1 mM GTP was clarified by centrifugation (50 000 r.p.m. for 10 min at 4°C) and polymerization was started at 37°C in the presence of 20 μ M taxol (15 min at 37°C). After at least 15 min, unpolymerized tubulin and GTP were removed by centrifuging the microtubules through a 40% sucrose cushion in ATPase buffer with 10 μ M taxol (50 000 r.p.m. for 10 min at 35°C). The microtubule pellet was rinsed and resuspended in a suitable volume of ATPase buffer supplemented with 10 μ M taxol. ATPase assays were performed on at least two preparations from two independent bacterial transformations for each construct.

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