Submolecular Domains of Bovine Brain Kinesin Identified by Electron Microscopy and Monoclonal Antibody Decoration

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Summary
Kinesin is a microtubule-activated ATPase thought to transport membrane-bounded organelles along MTs. To illuminate the structural basis for this function, EM was used to locate submolecular domains on bovine brain kinesin. Rotary shadowed kinesin appeared rod-shaped and ~80 nm long. One end of each molecule contained a pair of ~10 x 9 nm globular domains, while the opposite end was fan-shaped. Monoclonal antibodies against the ~124 kd heavy chains of kinesin decorated the globular structures, while those specific for the ~64 kd light chains labeled the fan-shaped end. Quick-freeze, deep-etch EM was used to analyze MTs polymerized from tubulin and cross-linked to latex microspheres by kinesin. Microspheres frequently attached to MTs by arm-like structures, 25-30 nm long. The MT attachment sites often appeared as one or two ~10 nm globular bulges. Morphologically similar cross-links were observed by quick-freeze, deep-etch EM between organelles and MTs in the neuronal cytoskeleton in vivo. These collective observations suggest that bovine brain kinesin binds to MTs by globular domains that contain the heavy chains, and that the attachment sites for organelles are at the opposite, fan-shaped end of kinesin, where the light chains are located.

Introduction
The nerve cell axon has proven to be an invaluable model system for studying mechanisms of intracellular organelle transport. There are bidirectional movements of numerous classes of membrane-bounded organelles in the axon (Nakai, 1955; Tytoll et al., 1981; Allen et al., 1982), and several theories have been proposed to explain the underlying molecular mechanisms (reviewed by Grafstein and Forman, 1980; Hammerschlag and Brady, 1988). Electron microscopic studies of the neuronal cytoskeleton in vivo have suggested that microtubules (MTs) and cross bridges between MTs and membranous organelles form the structural basis for this class of motility (Smith, 1971; Hirokawa, 1982; Miller and Lasek, 1985; Hirokawa and Yorifuji, 1986). Indeed, real-time observations of organelle movements in isolated axoplasm by video-enhanced light microscopy have demonstrated that bidirectional organelle translocation occurs along filaments that correspond to MTs (Brady et al., 1982, 1985; Schnapp et al., 1985; Vale et al., 1985a).

The observation that a nonhydrolyzable ATP analog, 5'-adenylimidodiphosphate (AMP-PNP), halted such movements and causes immotile organelles to bind stably to MTs in isolated axoplasm (Lasek and Brady, 1985) led to the discovery in neural tissue of a new protein whose binding to MTs is also stimulated by AMP-PNP (Brady, 1985; Vale et al., 1985a). This protein, kinesin, fulfills several of the properties expected of a motor molecule responsible for the transport of membrane-bounded organelles along MTs. For example, in cultured cells, kinesin is localized on vesicle-like structures that are codistributed with MTs throughout the cytoplasm (Pfister et al., 1989). In addition, kinesin isolated from a variety of sources can bind to MTs, hydrolyze ATP in a manner stimulated by MTs, and, in the course of doing so, generate plus-end directed forces along those MTs (Brady, 1985; Vale et al., 1985b, 1985c; Scholey et al., 1985; Kuznetsov and Gelfand, 1986; Cohn et al., 1987; Murofushi et al., 1988; Saxton et al., 1988; Wagner et al., 1989). To account for these properties, kinesin must contain binding sites for membrane-bounded organelles, ATP and MTs. In order to gain insight into the molecular basis of these functional domains, a number of recent studies have been aimed at dissecting the structure of kinesin.

In particular, determination of the quaternary structure of bovine brain kinesin has provided a framework for explaining the functions of the protein in terms of its molecular design (Bloom et al., 1988; Kuznetsov et al., 1988). The native protein contains two heavy chains of 120-124 kd and two light chains in the size range of 62-64 kd. Further analysis has indicated that bovine brain kinesin exists in solution as a highly elongated molecule, with an axial ratio of 20:1 or greater (Bloom et al., 1988), and that multiple forms of the heavy and light chains exist (Pfister et al., 1989; Wagner et al., 1989).

The structural studies just cited constitute a foundation on which to evaluate functions served by the kinesin heavy and light chains, and to establish how the holoenzyme is physically constructed from its constituent subunit polypeptides. The heavy chains, for example, evidently contain the binding sites for ATP and MTs (Gilbert and Sloboda, 1986; Penningroth et al., 1987; Bloom et al., 1986; Ingold et al., 1988; Yang et al., 1988; Kuznetsov et al., 1989). Scant progress had been made, though, toward either understanding the organization of heavy and light chains within the kinesin molecule, or the functions of the light chains.

To address these issues, we took advantage of the fact that the quick-freeze, deep-etch, and low angle, rotary shadowing methods of electron microscopy (EM) can be used for analyzing the submolecular structure and bio-
chemical nature of cytoskeletal components both in vivo and in vitro (Heuser and Salpeter, 1979; Tyler and Branton, 1980; Hirokawa, 1982; Hirokawa et al., 1984; Hirokawa and Hisanaga, 1987). Using these two approaches, we undertook studies aimed at establishing a more refined and detailed picture of the structure of kinesin. Bovine brain kinesin at >90% purity (Wagner et al., 1989) was utilized for all of the in vitro experiments. First, low angle, rotary shadowing was used for quantitative morphometric analysis of the kinesin molecule. Next, kinesin that had been decorated with purified monoclonal antibodies to kinesin heavy or light chains (Pfister et al., 1989) was rotary shadowed and isolated. Finally, complexes of kinesin, microtubules, and latex microspheres were analyzed by quick-freeze, deep-etch electron microscopy. These experiments were performed using only highly purified preparations of kinesin, tubulin, and monoclonal IgG antibodies (Figure 1A). As demonstrated by immunoblotting (Figure 1B), two heavy chain–specific anti-kinesins (H1 and H2) were used, as well as two monoclonals directed against the kinesin light chains (L1 and L2). Each of these antibodies recognizes a distinct kinesin epitope (Pfister et al., 1989).

Kinesin molecules processed for low angle, rotary shadowing exhibited a number of consistent morphological features, as can be seen in the low magnification field illustrated in Figure 2. Most of the molecules were approximately rod-shaped, between 50 and 100 nm in length, and contained structurally distinct domains at each end. Those features are documented in greater detail in the higher magnification images shown in Figure 3. One end of each molecule typically displayed one or two globular structures, referred to hereafter as "heads." The opposite end, or "tail," of the molecule was usually occupied by a fan-like structure. Often, two or more fine filamentous projections were seen to be part of the tail region, which had a flatter overall appearance than the heads. Connecting the head and tail regions was a long shaft. While the shaft on the majority of replicas appeared relatively straight, it was occasionally seen to be bent at a hinge-like region located near the middle of the molecule. As shown in the bottom row of Figure 3, the presence of this hinge was especially evident in samples that had been dissolved in low salt buffer (0.1 M ammonium acetate; see also Hisanaga et al., 1989).

The dimensions of morphologically distinct domains on kinesin molecules were measured, and the results are presented quantitatively in Figure 4. The overall length of the molecule was measured to be 80.4 ± 8.1 nm. The long and short diameters of the head were found to be 10.0 ± 1.9 and 8.6 ± 2.1 nm, respectively. A width of 14.7 ± 2.6 nm was determined for the tail region. The position of the hinge occasionally found within the shaft region was also measured, its location typically having been about 35 nm from the near edge of the globular heads (data not shown).
Figure 5 summarizes a series of experiments, in which two monoclonal antibodies to kinesin light chains were used to determine the positions of the corresponding epitopes on the native kinesin molecule. The top row demonstrates the appearance of normal mouse IgG, which was morphologically identical to all of our monoclonal anti-kinesins (not shown). The next row shows kinesin that had been incubated with a sample of the identical IgG preparation. Note that the kinesin molecules shown here are essentially indistinguishable from those displayed in Figure 3, indicating the absence of binding to kinesin by the irrelevant antibodies. Rows 3–5 in Figure 5 illustrate kinesin decorated with L1, while the final four rows show kinesin labeled with L2. Each of these light chain–specific antibodies labeled ~50% of the kinesin molecules, and the decorations consistently occurred at the fan-shaped ends of the molecules. The conclusion about the site of labeling by L1 and L2 is based on three considerations. First, the fan-shaped tails were not visible on kinesin molecules that were decorated by antibodies. Next, one end of each kinesin molecule had the characteristic appearance and dimensions of the globular heads. Finally, the opposite ends contained globular decorations that were in the size range of mouse IgG, larger than the globular heads of kinesin, and smaller than the sum of globular kinesin heads plus IgG. The size of the antibody decorations was usually consistent with the presence of one or two IgG molecules. Presumably, this reflected the binding of antibody to one or both of the light chain subunits associated with each molecule of bovine brain kinesin (Bloom et al., 1988; Kuznetsov et al., 1988).

Analogous data were obtained using heavy chain–specific anti-kinesins, as shown in Figure 6. This gallery of micrographs compares kinesin that was incubated with normal mouse IgG (row 1) and kinesin that was labeled with the H1 (rows 2–5) or H2 antibodies (rows 6–8). Again, each of the anti-kinesins decorated about half of the kinesin molecules, but the ultrastructure of these immunocomplexes was very distinct from those formed in the presence of light chain–specific monoclonals (compare Figures 5 and 6). The fan-shaped tails of kinesin could be clearly identified on molecules decorated by H1 or H2, but the globular head regions appeared unusually large. These images are consistent with the heavy chain epitopes recognized by H1 and H2 as being located on or near the globular heads of kinesin. As was found for the light chain–specific antibodies, the decorations formed on each kinesin molecule by H1 or H2 were in the size range of one or two IgG molecules. This is not surprising, considering that two heavy chains are present in the bovine brain kinesin molecule (Bloom et al., 1988; Kuznetsov et al., 1988) and that the extent of antibody binding could be variable.

To determine which portion of the kinesin molecule is
Figure 3. High Magnification View of Rotary Shadowed Kinesin
Several, representative molecules are shown here. One or two globular domains are visible at the right end of each molecule, while less well-defined, but generally fan-shaped, elaborations can be seen at the opposite ends. The bar in the lower right panel equals 100 nm.

responsible for binding MTs, purified tubulin was assembled with taxol, and mixed with AMP-PNP, kinesin, and 50 nm carboxylated, latex microspheres. These suspensions were then centrifuged, resuspended in small volumes and analyzed by the quick-freeze, deep-etch method of EM. As can be seen in Figure 7, microspheres often appeared to be cross-linked to MTs by 25–30 nm long, rod-like molecules, which frequently contained one or two conspicuous bulges where they contacted MTs. The diameter of each bulge was ~10 nm, and neither cross bridges between MTs and microspheres, nor MT-bound bulges, were observed when kinesin was omitted from the samples (data not shown). These findings, combined with those derived from the antibody decoration experiments (see Figures 5 and 6), strongly suggest that the globular heads, where two heavy chain epitopes are located, comprise the MT binding sites on kinesin.

Spinal cords were also processed by the quick-freeze, deep-etch method and observations were made of their neurites (Figure 8), where large numbers of membrane-bound organelles are transported rapidly and continuously along MTs. The purpose here was to compare the morphologies of cross-links between MTs and organelles in vivo with the cross-links formed by kinesin between MTs and synthetic microspheres in vitro. Some of the neuronal cross bridges were strikingly similar in appearance to those produced by kinesin molecules in the reconstituted system (compare Figures 7 and 8). Although we cannot at present be certain that any of the neuronal cross-links actually represent kinesin, it is significant that some of them, like those composed of kinesin in vitro, were ~25 nm long and contained a pair of globular bulges at their points of contact with MTs. Other approaches, such as immuno-EM of kinesin in vivo, will ultimately be required to determine whether such cross-links are, indeed, kinesin.
Discussion

The observations reported here have made it possible to construct a rational model that describes the functional properties of bovine brain kinesin in terms of submolecular structure (Figure 9). The overall morphology of the protein was demonstrated to be rod-like, but each end of the molecule was characterized by its own distinct type of structural elaboration. A pair of globular heads located at one end were shown to function as attachment sites for MTs and to contain two distinct antigenic sites of the kinesin heavy chain. The fan-shaped tail at the opposite end of kinesin was found to contain two light chain epitopes, and the available evidence strongly suggests that this tail serves as the binding site for the membrane-bounded organelle.

A hinged region was occasionally observed approximately halfway between the head and tail. The presence

Figure 4. Morphometric Analysis of Kinesin
The total length, tail width, and long and short diameters of the heads were measured for numerous kinesin molecules, like those shown in Figure 3. The size distribution for each parameter is summarized here.
Figure 5. Monoclonal Antibodies to Kinesin Light Chains Decorate the Fan-Shaped Tails of Kinesin

Samples of normal mouse IgG (top row) and kinesin that had been incubated with normal mouse IgG (row 2), L1 (rows 3-5), and L2 (rows 6-9) were processed for low angle, rotary shadowing. Note that normal mouse IgG failed to decorate kinesin (compare row 2 with Figure 3), but that the anti-light chain antibodies, L1 and L2, labeled the fan-shaped tails of the molecules. The bar in the lower right panel represents 100 nm.
Figure 6. Monoclonal Antibodies to Kinesin Heavy Chains Decorate the Globular Head Regions of Kinesin

Samples of kinesin that had been incubated with normal mouse IgG (top row), H1 (rows 2–5), and H2 (rows 6–8) were processed for low angle, rotary shadowing. Note that normal mouse IgG failed to decorate kinesin, but that the anti-heavy chain antibodies, H1 and H2, labeled the globular heads of the molecules. The bar in the lower right panel represents 100 nm.

of this hinge might explain why the total length of kinesin was measured to be ~80 nm, but the protein appeared to be only about one-third as long when cross-linking MTs to latex microspheres. As postulated in Figure 9A, perhaps a portion of the shaft of kinesin, extending from the hinge to the fan-shaped tail, accommodates the membrane-bounded organelle or microsphere. Consistent with this idea is the observation of similarly sized cross bridges between MTs and vesicles in vivo (Hirokawa, 1982; Miller and Lasek, 1985; Hirokawa and Yorifuji, 1986). It is also tempting to speculate that the hinge marks a point at which kinesin can bend, and that such a conformational change is related to ATP hydrolysis and organelle motility along MTs, or the regulation of those processes.
Figure 7. The Globular Heads of Kinesin Contain the Microtubule Binding Sites
Fifty nanometer carboxylated, latex microspheres were cross-linked by kinesin to microtubules made from pure tubulin and taxol, and the samples were analyzed by the quick-freeze, deep-etch method of EM. Three pairs of stereo images are shown here. Note that several kinesin cross bridges contain globular structures at their attachment sites for microtubules (see arrows). The size and appearance of these structures are consistent with the globular heads seen in rotary shadowed samples of kinesin (see Figure 3). The bar in the lower left panel equals 100 nm.
A number of structural and functional features of kinesin invite a comparison with myosin (see Figure 9C). Both proteins are long, rod-like molecules that contain heavy and light chain subunits, and a pair of globular heads, where polymers of cytoskeletal proteins can attach. These polymers stimulate a Mg\textsuperscript{2+}-ATPase activity associated with kinesin and myosin, and are mechanochemical partners of the enzymes. It is likely that the principal form of work performed intracellularly by kinesin is to move membrane-bounded organelles. Although myosin functions mainly as a contractile protein, it too appears able to be an organelle transport motor in a few cell types (Adams and Pollard, 1986; Warrick and Spudich, 1987). Contrasting these similarities, though, are numerous profound differences between kinesin and myosin. First, the mechanochemical partner of kinesin is the MT, while that of myosin is F-actin. Second, the order of the various steps in the mechanochemical cycle of kinesin apparently differs from that used by myosin (Lasék and Brady, 1985; Hackney, 1988). Third, most forms of myosin are nearly twice as long as bovine brain kinesin. Next, the light chains of kinesin appear to be located at the tail end of the molecule (see Figure 5), whereas the myosin light chains are part of the globular heads (Flicker et al., 1983; Yamamoto et al., 1985). Finally, there has been no indication yet that kinesin and myosin are related immunologically or by primary structure.

The contrasts between kinesin and dynein, the two MT-based mechanochemical ATPases, are equally pronounced. Dyneins are complex, multimeric proteins found in flagellar axonemes, where they are known to cause sliding between adjacent MTs and in the cytoplasm, where they have been proposed to be responsible for moving membrane-bounded organelles along MTs (Johnson, 1985; Lye et al., 1987; Paschal et al., 1987; Paschal and Vallee, 1987; Euteneuer et al., 1988; Gibbons, 1988). The cytoplasmic and axonemal dyneins consist of either two or three globular domains of 10–15 nm diameter each,
Figure 9. Models for the Substructure of Bovine Brain Kinesin

(A) Based on the data summarized in Figures 1-6, bovine brain kinesin appears to contain a pair of globular heads that bind microtubules and a fan-shaped tail involved in binding to membrane-bounded organelles or synthetic microspheres. A hinged region was found near the center of the molecule, and the tailward side of the hinge may rest against the surface of the organelle or microsphere. This could explain why cross bridges between microtubules, and either organelles or microspheres, are ~25 nm, while the overall length of kinesin is ~80 nm.

(B) Shown here is a scale model for the structure of kinesin that takes into account the diameters of the two globular head domains, the length of the central shaft region, the position of the hinge within the shaft (arrow), the width of the tail, and the apparent locations of each of the two heavy and light chains. The degrees to which the heavy and light chains extend within the shaft represent estimates, but all other details are based on the data shown in Figures 1-7.

(C) The morphological similarities between kinesin and myosin are superficial. Both kinesin and myosin are long, rod-shaped molecules with a pair of globular head domains, and heavy and light chain subunit polypeptides. As can be seen, though, kinesin contains a fan-shaped tail, which myosin lacks: the light chains of kinesin, but not myosin, are found at the tail end of the molecule; and kinesin is substantially shorter than most myosins.

The overall length of the dynein complex is 35-50 nm, considerably shorter than kinesin. In summary, kinesin, dynein, and myosin may be regarded as proteins that share some gross structural and functional features essential for performing work, but appear to be unrelated to one another in molecular terms.

A previous report on the ultrastructure of porcine brain kinesin indicated that the protein is a long, rod-shaped molecule with morphologically distinct ends, but the accompanying model is at odds with our data for the equivalent bovine brain enzyme (Amos, 1987). The porcine protein was described as being ~100 nm long and containing a small, bifurcated fork-like structure at one end of the molecule. This fork, along with a substantial length of the shaft region, was hypothesized to be involved in MT binding. In contrast, bovine brain kinesin was found to be only ~60 nm long (see Figures 2-4). In addition, we never observed a fork-like region, but consistently noted the presence of a pair of globular domains at one end of each kinesin molecule (see Figures 2 and 3). As shown in Figure 7, these globular heads alone apparently correspond to the MT binding site. Finally, at the opposite end of the molecule, porcine brain kinesin was envisioned as containing an elaborate, multibranched structure that attaches the molecule to the organelle, whereas our data do not support such a detailed model (compare Figure 9 here with Figure 6 in Amos, 1987).

Besides characterizing the overall morphology of bovine brain kinesin, the present study also supplies the first evidence for how both subunit polypeptides of kinesin are organized in the holoenzyme, and relates those findings to the physical locations of functional domains. Here, we have shown that a pair of heavy chain epitopes and the MT binding sites are on the globular heads of bovine brain kinesin, while two light chain domains are located on the fan-shaped tail at the opposite end of the molecule.

Moreover, our data strongly imply that the binding sites for membrane-bounded organelles are in the tail region, and that heavy and light chains are involved in the complementary functions of MT and organelle binding, respectively. The overall morphology of kinesin from other sources that have been examined very recently is consistent with that of the bovine brain enzyme (Hisanaga et al., 1989; Scholey et al., 1989). Furthermore, molecular biological and immuno-EM studies concurrent with those described here have also indicated that the globular heads of kinesin contain the MT-binding sites (Scholey et al., 1989; Yang et al., 1989, see accompanying paper). It is anticipated that these advances in illuminating the structure of kinesin will promote an improved understanding of the functional, biochemical, enzymatic, and molecular biological properties of the protein.

Experimental Procedures

Purification of Kinesin, Tubulin, and Monoclonal Antibodies

Kinesin was purified from bovine brain as described in detail previously (Wagner et al., 1989). The purity of the final product ranged from...
90%–95%, as judged by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie brilliant blue R250. Tubulin was purified from porcine or bovine brain using Phosphocellulose (Whatman, Clifton, NJ) or DEAE-Sepahdex chromatography (Pharmacia, Piscataway, NJ), as described earlier (Weingarten et al., 1975; Hirokawa et al., 1986; Bloom et al., 1988). Four extensively characterized monoclonal antibodies to kinesin (Pfister et al., 1989) were employed for the present study. Two of these, H1 and H2, recognize the major kinesin heavy chain, while the other antibodies, L1 and L2, react with the major light chain. H2 and L1 are also immunoreactive with minor forms of the heavy and light chains, respectively. Each of the antibodies was purified out of ascites fluid as a homogeneous IgG subisotype by Protein A-Sepharose (Pharmacia) or Protein A-affi-Gel (Bio-Rad; Richmond, CA) chromatography (Pfister et al., 1989). Prior to use, all purified proteins were dialyzed into PEM buffer (0.1 M PIPES (pH 8.8), 1 mM EGTA, 1 mM MgQ). SDS–PAGE and immunoblotting were performed as described (Pfister et al., 1989).

Low Angle, Rotary Shadowing EM

Ammonium acetate and glycerol were added to the protein solutions to final concentrations of 0.5 M (occasionally 0.1 M) and 50% (by volume), respectively. For antibody decoration experiments, this step was completed after mixtures of kinesin at -50 μg/ml and a 2-fold molar excess of antibody had incubated together for 1 hr at room temperature. Protein solutions supplemented with ammonium acetate and glycerol were sprayed onto freshly cleaved mica flakes, which were subsequently dried under vacuum as described previously (Tyler and Brandton, 1980; Hirokawa, 1985). Rotary shadowing with platinum was performed at an angle of 6° using a Balzers (Hudson, NH) model 301 freeze-fixture apparatus, and the replicas were processed further as described previously (Hirokawa et al., 1988).

The dimensions of various domains on kinesin molecules were determined by a quantitative morphometric method. Micrographs were printed at a magnification of 140,000x. Then, length measurements were made by using a magnifying glass to examine profiles of kinesin, along side of which a ruler was placed. All specimens were observed and photographed on a JEOL 2000 EX electron microscope at 100 KV.

Quick-Freeze, Deep-Etch EM

One hundred micrograms each of kinesin and tubulin were incubated for 15 min at 37°C in the presence of 1 mM 5'-adenylylimidodiphosphate (AMP-PNP), 1 mM GTP, 20 PM taxol (gift from Matthew Suffness, 677 Clifton, NJ), or DEAE-Sepahdex chromatography (Pharmacia; Piscataway, NJ), as described earlier (Weingarten et al., 1975; Hirokawa et al., 1986). Bloom et al., 1988). Fast axonal transport in squid giant axon. Science 218, 1127–1128.


