The Shapes of the Motor Domains of Two Oppositely Directed Microtubule Motors, ncd and Kinesin: A Neutron Scattering Study

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ABSTRACT The shapes of the motor domains of kinesin and ncd, which move in opposite directions along microtubules, have been investigated. Using proteins expressed in *Escherichia coli*, it was found that at high salt (>200 mM) *Drosophila* ncd motor domain (R335-K700) and human kinesin motor domain (M1-E349) were both sufficiently monomeric to allow an accurate determination of their radii of gyration (R_g) and their molecular weights. The measured R_g values of the ncd and kinesin motor domains in D_2O were 2.06 ± 0.06 and 2.05 ± 0.04 nm, respectively, and the molecular weights were consistent with those computed from the amino acid compositions. Fitting of the scattering curves to ~3.5 nm resolution showed that the ncd and kinesin motor domains can be described adequately by triaxial ellipsoids having half-axes of 1.42 ± 0.38, 2.24 ± 0.44, and 3.65 ± 0.22 nm, and half-axes of 1.52 ± 0.23, 2.00 ± 0.25, and 3.73 ± 0.10 nm, respectively. Both motor domains are described adequately as somewhat flattened prolate ellipsoids with a maximum dimension of ~7.5 nm. Thus, it appears that the overall shapes of these motor domains are not the major determinants of the directionality of their movement along microtubules.

INTRODUCTION

There are a large number of kinesin-related motor proteins (see, e.g., Goodson et al., 1994). These proteins, which form a kinesin superfamily, play diverse roles in cell function, such as organelle transport and cell division. Within this superfamily, the non-claret disjunctional protein, or ncd, is of particular interest. Unlike kinesin, the "motor domain" of ncd occurs at the C terminus, yet the ncd motor domain has a 41% sequence identity to the kinesin motor domain (Endow et al., 1990; McDonald and Goldstein, 1990). Also, unlike kinesin, ncd moves toward the minus-end of microtubules (McDonald et al., 1990; Walker et al., 1990).

Chandra et al. (1993) and Stewart et al. (1993) have shown that the motor domain portions of ncd and kinesin determine the direction of their movement along microtubules. To gain insight into the origin of this directionality, we compared the low-resolution structures of ncd and kinesin motor domains. The *Drosophila* ncd motor domain construct (R335-K700) and the human kinesin motor domain construct (M1-E349) were compared. Sequence alignment analysis (R. J. Fletterick, personal communication) showed that the primary difference between these constructs is not extra residues in the C- or N-termini, but rather that the ncd motor domain has two extra loops. These extra loops could alter the overall structure of the ncd motor domain.

We have used neutron solution scattering. Preliminary experiments showed that the ncd motor domain suffered measurable radiation damage after 2 min of exposure to synchrotron x-rays (our unpublished results). Thus neutron scattering, which produces virtually no radiation damage to proteins (Schoenborn, 1975), is more suitable than x-ray scattering for such fragile proteins. Further, neutron scattering measurements in a D_2O solvent allow one to measure the scattering of the samples at comparable or lower concentrations than is possible in x-ray scattering experiments. Also, proteins in D_2O have a high absolute scattering-length density contrast so that the data in the small-angle region are affected minimally by scattering-length density fluctuations within the protein (Ibel and Stuhrmann, 1975). Such high-contrast data are suitable for investigating the overall shapes of proteins. From an analysis of the neutron scattering patterns of the ncd motor domain and the kinesin motor domain, we found that these proteins have very similar shapes.

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MATERIALS AND METHODS

Expression and purification of the truncated ncd protein

Drosophila ncd motor domain was prepared as described previously (Sablin and Fletterick, 1995) with some modifications. The recombinant plasmid pHB40P-ncd containing the truncated ncd gene R335-K700 was transformed into BL21(DE3) host cells carrying the bacteriophage T7 RNA polymerase gene under the control of the IPTG-inducible *lac* UV5 promoter (Studier et al., 1990). Cells were grown in a 20-l fermenter (LH Fermentation Ltd., Bucks, U.K.) at 22°C and induced at OD_{600} 0.3 by addition of IPTG to 0.1 mM followed by vigorous stirring for 9–12 h at 22°C. Cell pellets from the 13-l culture were suspended to a final volume of 750 ml in 10 mM phosphate buffer (PB), pH 7.2, containing 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitors and frozen at −80°C. After thawing at 4°C, 100 ml of cell suspension was incubated for 1 h in the presence of 0.2 mg/ml FPLC-purified lysozyme (L-6876, Sigma Chemical Co., St. Louis, MO) and 0.5% Triton X-100, followed by incubation with 40 μg/ml DNase I and 10 mM $MgCl_2$ for 20 min. The cell lysate was centrifuged at 30,000 × *g* for 30 min. Supernatants were then loaded on a 115-ml S-Sepharose FF (Pharmacia Biotech, Uppsala, Sweden) column equilibrated in PB. The column was washed

extensively with PB, and a 1500-ml linear NaCl gradient (0.1–0.35 M NaCl) was used to elute the ncd motor domain. Fractions containing the ncd motor domain were pooled, concentrated by ultra-filtration, and dialyzed against 10 mM Tris-HCl buffer (TB), pH 7.5, containing 80 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitors. The dialyzed protein was passed through a 50-ml Q-Sepharose HP column (Pharmacia) equilibrated in TB. Pooled fractions containing the ncd motor domain were re-concentrated and dialyzed against a buffer (PSB) containing 50 mM PIPES, pH 7.2, 500 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, and protease inhibitors. Purified ncd motor domain was stored in 50% glycerol at −20°C (Lockhart and Cross, 1994).

Expression and purification of the truncated kinesin protein

Expression of human kinesin motor domain (M1-E349) was achieved using BL21(DE3) host cells transformed with plasmid pHB40P carrying this coding region. Cells were grown in shake flasks at 35–37°C for 16 h on LB media containing 75 μg/ml ampicillin. Harvested cells from 12.5 l of culture were suspended in 150 ml of the salt-free buffer (25 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM DTT, and protease inhibitors), sonicated, and pelleted. The supernatant of the lysed cells was loaded on a phosphocellulose column equilibrated in the salt-free buffer. The fractions containing the kinesin motor domain were eluted at 0.6–1.0 M KCl in a linear gradient of 0–1.0 M KCl. The pooled kinesin motor domain fractions were passed through a S-Sepharose HP column (Pharmacia) equilibrated in the salt-free buffer and loaded onto a Q-Sepharose HP column (Pharmacia). The fractions containing the kinesin motor domain, eluted with a linear gradient of 0–0.6 M KCl, were pooled. After dilution and further purification using a MonoQ column (Pharmacia) with a 0–0.45 M KCl gradient, the collected fractions were concentrated by ultra-filtration and dialyzed against a buffer containing 25 mM PIPES (pH 6.8), 200 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitors.

Biochemical methods

ATPase activities of ncd and kinesin motor domains were measured colorimetrically (White, 1982). Concentrations of proteins were measured by the Biuret method (Gornall et al., 1949). The extinction coefficients of 0.1% solutions of ncd and kinesin motor domains at 280 nm were estimated to be 0.62 and 0.63 cm^{-1}, respectively.

Sample preparation for neutron scattering experiments

Stored ncd motor domain was dialyzed against PSB to remove glycerol. The dialyzed protein was then concentrated and further purified by gel filtration using a 700-ml Sephacryl S-200 HR column (Pharmacia) equilibrated in PSB. For the kinesin motor domain, freshly prepared protein was used. Samples for neutron scattering experiments were prepared by dialysis against a buffer containing 50 mM PIPES, pH 7.0, 200–500 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 10 μM ADP, and 5 μM Ap5A in 98.5% D_2O. All samples were clarified by ultra-centrifugation at 150,000 × g for 1 h just before exposure to neutrons. Fig. 1 shows 15% SDS-PAGE patterns of ncd and kinesin motor domains.

Neutron scattering experiments

Most neutron scattering experiments were performed on the H9B small-angle spectrometer (Schneider and Schoenborn, 1984) of the High Flux Beam Reactor at Brookhaven National Laboratory (BNL). A wavelength (λ) of 0.508 nm was used with a fractional spread (Δλ/λ) of 10%. Data were collected with a two-dimensional ^{3}He-filled multi-wire detector at a sample-to-detector distance of 143.3 cm. With this setting, the observed range of the scattering vector s (=2 sin θ/λ, where 2θ is the scattering angle) was 0.028–0.45 nm^{-1}. Some data were taken using the NG3 30-m small-angle spectrometer (Hammouda et al., 1993) of the Cold Neutron Research Facility at the National Institute of Standards and Technology (NIST), with λ = 0.5 nm, Δλ/λ = 15%, and a sample-to-detector distance of 250 cm. The samples were maintained at 6°C in standard quartz optical cells (Hellma GmbH and Co., Müllheim/Baden, Germany) having a 5-mm path length. For the measurements of the kinesin motor domain, these cells were siliconized to prevent the proteins from adsorbing to the cell surface. Collected two-dimensional patterns were corrected for buffer scattering, normalized to beam intensity and sample transmission, and radially integrated to yield the net scattering intensity $I(s)$.

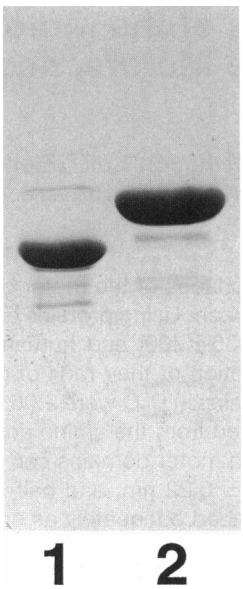

FIGURE 1 15% SDS polyacrylamide gel electrophoresis patterns of kinesin (*lane 1*) and ncd (*lane 2*) motor domains.

The scattering curves were analyzed using the Guinier approach (Guinier and Fournet, 1955), in which the small-angle region of the scattering curve is approximated by

$$I(s) = I(0)\exp(-4\pi^2 s^2 R_g^2/3),$$

where $I(0)$ is the scattering intensity at zero scattering angle and R_g denotes the radius of gyration. Thus, if $\ln(I(s))$ is plotted against s^2 (a "Guinier Plot"), the scattering curve is approximated by a straight line. The R_g and molecular weight can be estimated from the slope and the intercept ($I(0)$) of this straight line, respectively.

Molecular weights were estimated by the procedure described by Jacrot and Zaccai (1981) for a D_2O solvent. The $I(0)$ values measured at various protein concentrations were divided by the protein concentration c and extrapolated to $c = 0$. This extrapolated $I(0)/c$ obtained in 98.5% D_2O and the assumption that the scattering-length density of the proteins is matched out (i.e., $I(0)/c = 0$) at 42% D_2O, were then used to extrapolate the I(0)/c to 100% H_2O. The $I(0)/c$ in H_2O estimated in this way and experimental incoherent scattering from H_2O were used to estimate the molecular weight.

Modeling the scattering curves with triaxial ellipsoids

The experimental scattering curves were fit with model scattering curves expected from uniform-density triaxial ellipsoids. For all possible values of the three half-axes (A, B, and C) of an ellipsoid subjected to the constraints described below, scattering curves were computed by numerical integration of the analytical expression of the structure factor for an ellipsoid (Hiragi

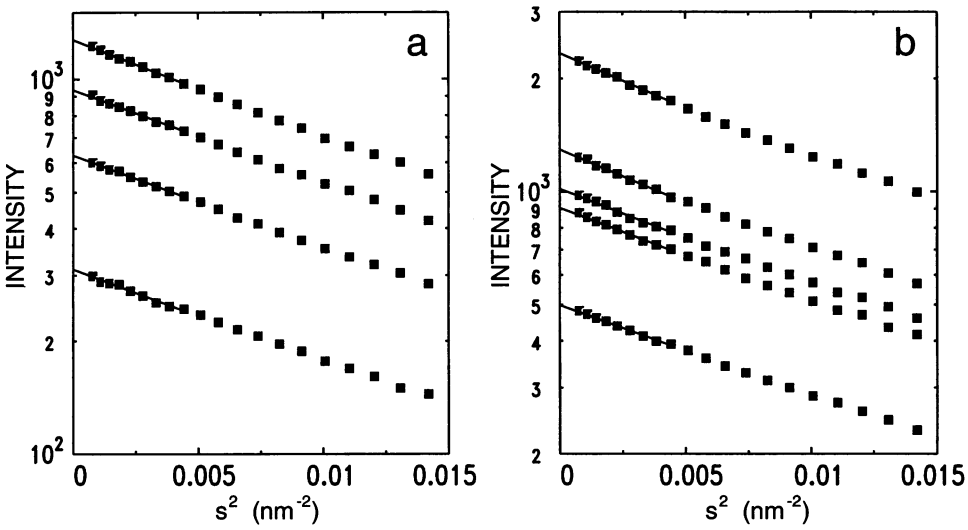

FIGURE 2 Guinier plots of the scattering curves at various protein concentrations. (*a*) Guinier plots of the ncd motor domain. The concentrations are, from top to bottom (in mg/ml), 8.84, 6.60, 4.29, and 2.19. (*b*) Guinier plots of the kinesin motor domain. The concentrations are, from top to bottom (in mg/ml), 16.3, 9.38, 7.42, 6.49, and 3.61. The scattering curves of the ncd motor domain were measured at 500 mM NaCl, whereas those of the kinesin motor domain were measured at 200 mM NaCl. Error bars are within the symbols.

and Ihara, 1981). Convolution of model scattering curves with neutron beam geometry and wavelength spread (Curmi et al., 1988) showed that these nonidealities negligibly altered the shape of the model curves in the fitting region. The goodness of fit was assayed by $\chi^2 = \sum_j \{(I_{exp}(s_j) - I_{model}(s_j))^2/\sigma_j^2\}$, where $I_{exp}(s_j)$ and $I_{model}(s_j)$ are the experimental and the model scattering curves at s_j, respectively, and σ_j is the SD at s_j. The model producing the lowest χ^2 was chosen as the best model.

The R_g and the volume of the protein were used as constraints. The R_g of the models was constrained to be within the range of 1 SD of the experimental R_g. The partial specific volumes of most proteins are within a range from 0.69 to 0.75 cm^3/g (Cantor and Schimmel, 1980). Thus, the volume was constrained to be within a 4% range of the volume calculated from the partial specific volume of 0.72 cm^3/g and the molecular weight calculated from amino acid composition.

RESULTS AND DISCUSSION

ATPase activities of the samples used for neutron scattering experiments were measured at 25°C. The ATPase rates were 0.0092 s^{-1} for the ncd motor domain and 0.019 s^{-1} for the kinesin motor domain. The value for the ncd motor domain is in agreement with the value (~0.01 s^{-1}) for a similar (but not identical) construct reported by Lockhart and Cross (1994) but somewhat lower than the values (0.03 s^{-1}) for other similar (but not identical) constructs reported by Chandra et al. (1993) and Stewart et al. (1993). The proteins used here were similar to preparations used in crystallization studies (Sablin and Fletterick, 1995). The ATPase rate of the ncd motor domain was stable for at least 2 weeks when stored at 6°C. The ATPase rate of the kinesin motor domain is consistent with the values reported for similar constructs from *Drosophila* kinesin (Stewart et al., 1993; Huang and Hackney, 1994). The ATPase activities of both the ncd and kinesin motor domains were unaffected by exposure to neutrons.

Fig. 2 shows the Guinier plots of the net scattering curves of ncd and kinesin motor domains at various concentrations. The curves in the region of $s^2 < 0.005$ nm^{-2} were well approximated by a straight line. No clear sign of significant aggregation, as would be indicated by an upward deviation from the straight-line fit in the small-angle region, is observed. To estimate R_g correctly, fitting was done in the region of $s < 1/2\pi R_g$ (Guinier and Fournet, 1955).

Fig. 3 shows $R_g(c)$ and $I(0)/c$ as a function of the protein concentration. For the ncd motor domain in 500 mM NaCl,

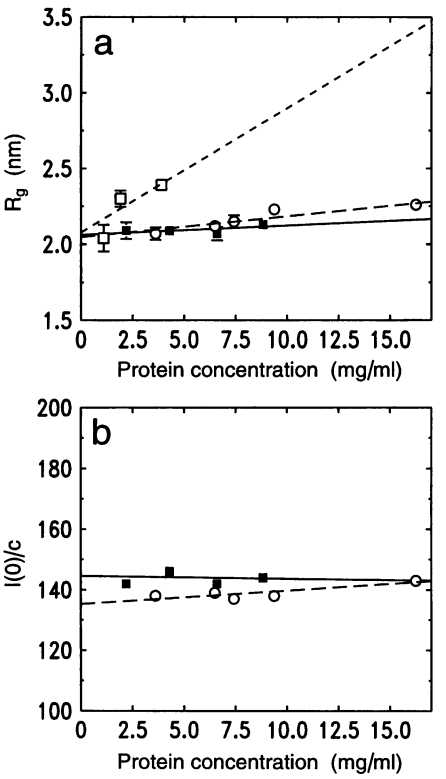

FIGURE 3 Protein concentration dependence of R_g and of $I(0)/c$. (*a*) R_g vs. the protein concentration c of the ncd motor domain with 500 mM NaCl present (■) and of the ncd motor domain with 130 mM NaCl present (□). The R_g values of the kinesin motor domain at 200 mM NaCl are also shown (○). (*b*) $I(0)/c$ plotted against c of the ncd motor domain at 500 mM NaCl (■) and of the kinesin motor domain at 200 mM NaCl (○). Error bars are within the symbols unless shown.

fitting the data points with a straight line (e.g., Pilz, 1982) results in almost zero slope, indicating that interparticle interference is negligible. The extrapolated $R_g(0)$ was 2.06 ± 0.06 nm. The extrapolated $I(0)/c$ value was used to estimate the molecular weight, which was found to be 40,500 ± 800. This is in good agreement with the molecular weight of 41,369 calculated from amino acid composition. These values, together with the negligible interparticle interference, indicate that the ncd motor domain exists as a monomer in 500 mM NaCl.

A high salt condition was chosen because we observed that at lower salt concentrations the $R_g(c)$ of the ncd motor domain exhibited a strong positive slope. For example, the R_g values at 130 mM are shown in Fig. 3 *a*. Such a positive slope is indicative of aggregation or association of the proteins. The $R_g(0)$ value at 130 mM NaCl was 2.08 ± 0.09 nm, and the $R_g(0)$ values estimated from other sets of data at low salt also fell near 2.0 nm. Thus, there is no large conformational change between low salt concentrations and 500 mM NaCl.

The scattering curves of the kinesin motor domain were measured at 200 mM NaCl. R_g and $I(0)/c$ versus concentration plots of the kinesin motor domain show a slight positive slope. This slope in the kinesin motor domain may reflect a behavior with varying salt concentration similar to that of the ncd motor domain. The $R_g(0)$ is 2.05 ± 0.04 nm, which is close to the value of the ncd motor domain. The molecular weight was estimated to be 38,000 ± 500, which is also in good agreement with the molecular weight of 39,263 calculated from amino acid composition. The molecular parameters of ncd and kinesin motor domains are summarized in Table 1. Note that the R_g values presented here were obtained in D_2O. Because the R_g depends slightly on contrast for proteins (see Ibel and Stuhrmann, 1975), the R_g values obtained here will be slightly different from the in vacuo R_g values obtained by crystallography. This, however, will not affect any of the conclusions of this work.

To obtain more shape information, fitting of the scattering curves with triaxial ellipsoids was performed. The scattering curves, divided by c, were extrapolated to $c = 0$ to eliminate any possible interparticle interference (Pilz, 1982). Such extrapolated curves were used for fitting. Fits were obtained over the range from $s = 0.07$ to 0.25 nm^{-1}. Fig. 4 shows the extrapolated scattering curves and the scattering curves of the best fitted triaxial ellipsoids. The values of the three half-axes of the best fitted ellipsoids are summarized in Table 1. The curves from the models are in good agreement with the experimental curves up to $s \approx 0.3$ nm^{-1}. The volumes of these ellipsoids are within a reasonable range of the volumes calculated from amino acid composition and the partial volume of each amino acid (Jacrot and Zaccai, 1981).

The model scattering curves deviate from the experimental curves beyond $\sim s > 0.3$ nm^{-1} (not shown). However, the scattering intensity from the proteins is so low in this region that the net scattering curve could be affected by small errors in buffer subtraction. Tests with slightly different normalization factors for buffer subtraction did not alter appreciably the scattering curves in the smaller-angle region used in the fitting. Thus, any such error has a negligible effect on the extracted structural parameters. It is concluded, therefore, that both ncd and kinesin motor domains are well represented by a triaxial ellipsoid at a resolution of ~3.5 nm. (The good fit of general ellipsoids to the data suggests that they are an adequate representation of the structure at low-resolution and that the long axes of the ellipsoids are a good estimate of the maximum chord. However, we cannot preclude the possibility that a small amount of mass protruding from the main portion of the structure would not be detected by solution scattering. In such a case, the maximum chords in these molecules would be greater than the long axes of the equivalent ellipsoids.) Shapes of ncd and kinesin motor domains are very similar and can be described as a prolate ellipsoid flattened somewhat in the direction of the smaller axis.

It is possible to compare the shapes obtained here with results from other measurements. Another ncd motor domain construct containing residues 333–700 was suggested to be "almost completely globular" from hydrodynamic measurements ($s_{20,w}$ and Stokes radius determined from FPLC gel filtration), whereas electron microscopic observation of rotary shadowed samples gave head dimensions of ~6 × 6.8 nm (Chandra et al., 1993). Similar hydrodynamic measurements for *Drosophila* kinesin motor domain (residues 1–340) yielded an axial ratio of 2.7 for an equivalent prolate ellipsoid of revolution (Huang et al., 1994). Eden and his colleagues (1995), using another hydrodynamic method, transient electric birefringence, measured an axial ratio of 2.5 for the ncd motor domain (R335-K700). However, they found that the kinesin motor domain (M1-E349) has a much longer relaxation time

TABLE 1 Summary of molecular parameters of ncd and kinesin motor domains

				Best fitting triaxial ellipsoids					
	$R_g^{*\ddagger}$ (nm)	m.w.*	Calculated m.w.§	$A^{\parallel}$ (nm)	$B^{\parallel}$ (nm)	$C^{\parallel}$ (nm)	Volume$^{\parallel}$ (nm^3)	χ^2_ν	Calculated volume§ (nm^3)
ncd (335–700)	2.06 ± 0.06	40,500 ± 800	41,369	1.42 ± 0.38	2.24 ± 0.44	3.65 ± 0.22	48.6 ± 2.6	0.80	52.1
kinesin (1–349)	2.05 ± 0.04	38,000 ± 500	39,263	1.52 ± 0.23	2.00 ± 0.25	3.73 ± 0.10	47.5 ± 1.4	1.50	49.4

*From the scattering data. SDs of errors propagated from counting statistics only are shown.
‡Measured in a D_2O solvent.
§From amino acid compositions.
‖The best values and 95.4% confidence limits of the parameters are shown.

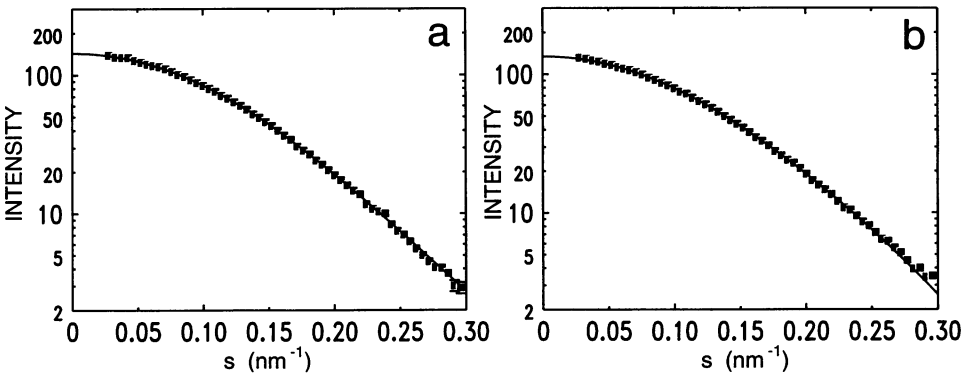

FIGURE 4 Fitting of the scattering by ncd (*a*) and kinesin (*b*) motor domains with triaxial ellipsoids. Extrapolated scattering curves ($I(s)/c$ vs. s) to $c = 0$ (■) and the scattering curves of the "best fitting" triaxial ellipsoids (——) are shown. The fitting region was from $s = 0.07$ to 0.25 nm^{-1}.

than the ncd motor domain. This suggested to these authors that the kinesin motor domain has a greater axial ratio than does the ncd motor domain. The hydrodynamic axial ratio of the ncd motor domain (2.5–2.7) is somewhat larger than found here. Also, as pointed out by Cantor and Schimmel (1980) and by Eden et al. (1995), it is difficult in general to distinguish between prolate and oblate ellipsoids from hydrodynamic measurements. On the other hand, as we have shown here, with neutron scattering experiments the three half-axes of an equivalent general ellipsoid can be determined unambiguously.

It is possible to compare the approximate shapes of ncd and kinesin motor domains with the nucleotide-free myosin subfragment one (S1) crystal structure (Rayment et al., 1993). If the volume of S1 is scaled to that of the ncd motor domain while retaining its shape, the R_g drops from 4.6 to 3.1 nm. (Here it is assumed that the partial specific volumes of the ncd motor domain and S1 are identical.) The large difference in the R_g implies that S1 is significantly more asymmetric than the ncd (or kinesin) motor domain. This is confirmed by wide-angle scattering data as well. Scattering intensities, normalized to one at $s = 0$, were plotted versus $s \times R_g$ to compare directly the shapes of the scattering curves (Fig. 5). It is seen that the scattering curves of ncd and kinesin motor domains are very similar but quite different from that calculated from the S1 crystal structure.

The overall shapes of ncd and kinesin motor domains are very similar. Thus, the directionality of these motors does not appear to arise from either their kinetics (Lockhart and Cross, 1994) or their low-resolution structure. Recent experiments (Hackney, 1994; Svoboda et al., 1994; Berliner et al., 1995; Gilbert et al., 1995) suggest cooperativity of the two motor domains in a single molecule. The maximum dimension of these motor domains, ~7.5 nm, together with a kinesin step size of 8 nm (Svoboda et al., 1993), accords well with a "hand-over-hand" mechanism of the movement. However, the observation that single-headed kinesin frequently fails to track properly along a protofilament, yet still exhibits net longitudinal movements on microtubules (Berliner et al., 1995), implies that even if the cooperativity of the two motor domains plays an important role, the determinants of the directionality are within a single motor domain. One simple explanation for the differing directionalities of these motor domains may be their different polarities. Assume, for example, that the localized microtubule-binding regions in, say, the ncd motor domains are significantly "twisted" relative to those of the kinesin motor domains. The orientation of the ncd motor domain relative to the microtubule axis would then be different from that of the kinesin motor domain, even if the interactions with microtubules were locally the same. The power stroke then could move these motor domains in opposite directions. Alternatively, ncd and kinesin motor domains might interact with different tubulin subunits in microtubules and thereby confer directionality (Endow, 1995). High-resolution structures of these motor domains by x-ray crystallography (Sablin and Fletterick, 1995) should provide important insights into this problem. It will also be important to obtain structural information about these motor proteins bound to microtubules in various states. In addition to crystallography and electron microscopy, neutron scattering, which has the capability of rendering only particles of interest "visible," may be suitable for this purpose. Special methods in neutron scattering (Fujiwara and Mendelson, 1994), such as the triple isotopic substitution method and the statistical labeling method, could be used to investigate the shapes of

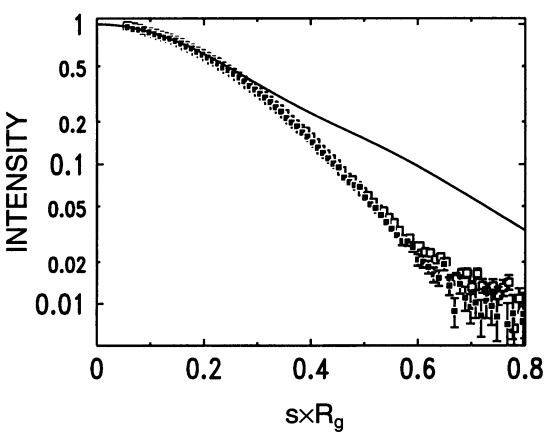

FIGURE 5 Plots of the scattering intensities vs. $s \times R_g$. The experimental scattering curves from the ncd motor domain (■) and from the kinesin motor domain (□), extrapolated to $c = 0$, and the scattering curve calculated from the crystal structure of S1 (——) are shown. Each scattering curve is normalized so that $I(0) = 1.0$.

these motor proteins bound to microtubules and their orientations relative to filament axis.

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