Influence of the Kinesin Neck Domain on Dimerization and ATPase Kinetics*

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Motor domains of kinesin were expressed that extend from the N terminus to positions 346, 357, 365, 381, and 405 (designated DKH346–DKH405) to determine if the kinetic differences observed between monomeric DKH340 and dimeric DKH392 (Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6865–6869) were specific to these constructs or due to their oligomeric state. Sedimentation analysis indicated that DKH346, DKH357, and DKH365 are predominantly monomeric and that DKH381 and DKH405 are predominantly dimeric at 0.01–0.03 μM, the concentrations used for ATPase assays. In buffer with 25 mM KCl, all have high kcat values of 38–96 s⁻¹ at saturating microtubule (MT) levels. Monomeric DKH346, DKH357, and DKH365 have Kcat(MT) values of 17, 9, and 1.4 μM, respectively, but the Kcat(MT) values for the dimeric species are significantly lower, with 0.02 and 0.14 μM for DKH381 and DKH405, respectively. The three new monomers release all of their ADP from the N terminus to positions 346, 357, 365, 381, and 405 (designated DKH346–DKH405) to determine if the kinetic differences observed between monomeric DKH340 and dimeric DKH392 (Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6865–6869) were specific to these constructs or due to their oligomeric state. Sedimentation analysis indicated that DKH346, DKH357, and DKH365 are predominantly monomeric and that DKH381 and DKH405 are predominantly dimeric at 0.01–0.03 μM, the concentrations used for ATPase assays. In buffer with 25 mM KCl, all have high kcat values of 38–96 s⁻¹ at saturating microtubule (MT) levels. Monomeric DKH346, DKH357, and DKH365 have Kcat(MT) values of 17, 9, and 1.4 μM, respectively, but the Kcat(MT) values for the dimeric species are significantly lower, with 0.02 and 0.14 μM for DKH381 and DKH405, respectively. The three new monomers release all of their ADP on association with microtubules, whereas the two new dimers retain approximately half of their ADP, consistent with the half-site reactivity observed previously with dimeric DKH392. Both the kcat(ADPase)/Kcat(MT) values for stimulation of ATPase by MTs and the kcat(ADP)/Kcat(ADP) values for stimulation of ADP release by MTs were determined in buffer containing 120 mM potassium acetate. The ratio of these rate constants (kcat(ADPase)/kcat(ADP)) is 60–100 for the dimers, indicating hydrolysis of many ATP molecules per productive encounter with a MT as observed previously for DKH392 (Hackney, D. D. (1995) Nature 377, 448–450). For the monomers, kcat(ratio) values of ~4 indicate that they also may hydrolyze more than one ATP molecule per encounter with a MT and that the mechanism of hydrolysis is therefore fundamentally different from that of actomyosin. DKH340 is an exception to this pattern and may undergo uncoupled ATP hydrolysis.

Kinesin is a molecular motor that is capable of producing movement along microtubules (see Refs. 1–3 for review). A striking feature of its motility is that a single molecule of native dimeric kinesin is able to attach to a microtubule (MT) and slide along it without net dissociation (4–8). This is in contrast to the movement of myosin along actin, which is believed to be processive.

The N-terminal 340 amino acids of kinesin are conserved among all superfamily members and contain the sites for ATP hydrolysis and interaction with MTs. The structure of this domain has recently been solved by x-ray crystallography (9). The region between amino acids 340 and 400 is predicted to be α-helical and is likely to contain a region of coiled-coil (10, 11). Previous work has established that DKH340 (containing amino acids 1–340) is monomeric, with an s20,w value of 3.3 S, whereas DKH392 (containing amino acids 1–392) is dimeric, with an s20,w value of 5.2 S (11). This led to a revised domain model of kinesin in which the two minimal motor domains are dimerized through interaction of the coiled-coil region in the neck without a highly flexible hinge at their point of attachment to the neck (11). The poorly conserved region around position 400 is not predicted to readily form a coiled-coil structure, and it likely serves as a flexible hinge that attaches a functional dimer head unit to the rest of the coiled-coil stalk (12). Subsequent determination of the oligomeric state of constructs of different lengths and from other species has confirmed this pattern and refined the region required for dimerization (7, 8, 13, 14). A high degree of processive behavior likely requires the coordination of two head domains in a dimer as only dimeric constructs of kinesin can track in a linear path down a MT (7).

The various head constructs of kinesin have a wide range of reported ATPase properties (7, 8, 10, 15–19). This reflects both the intrinsic differences between constructs due to their length, oligomeric state, and species of origin and differences in assay conditions, particularly the ionic strength and the concentration of heads. The work presented here is an examination of seven head constructs of Drosophila kinesin that differ in the amount of the neck domain that is included. This extensive series of constructs allows systematic evaluation of the influence of the length of the neck on the oligomeric state as well as evaluation of the influence of dimerization on kinetic properties under uniform assay conditions. These constructs exhibit a large range of properties, which suggests that caution must be exercised to prevent overinterpretation of results with a more limited series. Despite this variation, however, several characteristic differences between dimers and monomers have emerged.

EXPERIMENTAL PROCEDURES

The plasmids pDKH346, pDKH357, pDKH365, pDKH381, and pDKH405 were obtained by polymerase chain reaction with the proof-reading Pfu polymerase essentially as described for pDKH340 and pDKH392 (11, 20). Given the wide range of kinetic properties observed here with head constructs that all contain the same catalytic domain, it is reasonable to be concerned that differences may result from spurious mutations that were introduced in some constructs during cloning. All of the constructs used here were obtained by subcloning into the same pET1 site corresponding to amino acid 295) was derived by polymerase chain reaction, and the DNA sequence of the polymerase chain reaction-derived region was determined and shown not to contain any errors. DNA sequences were obtained by the DNA Sequencing Facility of the
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Table I
Sedimentation coefficients of head domains

| Head construct | 0.02 μm² | 0.3 μm² | 1.0 μm² | 20.0 μm² | 120 mM KCl, 20 μm² |
|----------------|---------|---------|---------|---------|--------------------|
| DKH340         | ND⁵     | 3.5     | (3.0)   | 3.6     | ND                 |
| DKH346         | ND⁵     | 3.6     | 3.6     | 3.6     | ND                 |
| DKH357         | ND⁵     | 3.6     | 3.6     | 3.6     | ND                 |
| DKH385         | ND⁵     | 3.5     | 3.5     | 3.7     | 3.6                |
| DKH381         | 5.1     | 5.2     | 5.7     | 8.0     | 5.7                |
| DKH392         | ND⁵     | 5.1     | (5.2)   | 6.1     | 5.2                |
| DKH405         | 5.1     | 5.1     | 5.4     | 6.3     | 5.2                |

⁴ Initial head concentration.
⁵ ND, not determined.

Sucre density centrifugation was performed as described under “Experimental Procedures” in A25 buffer with 0.1 mM ATP and 25 mM KCl or 120 mM potassium acetate.

University of Pittsburgh by the dye terminator method. As a further check for possible introduction of mutations into the N-terminal region during subcloning, the PstI tail fragment of the clone of pDKH357 that was used for most of the protein preparations was excised and religated into a pBK392 backbone. DKH357 protein expressed by this new construct had the same high Kₐ₅₅₅₅ values as the original DKH357 clone, indicating that the difference in Kₐ₅₅₅₅ between DKH357 and DKH392 is not due to unintended changes in sequence in the catalytic region.

Preparations of the head domains proteins used in this work (including new preparations of DKH392 and DKH340) were performed essentially as described previously for DKH340 and DKH392 (11, 20), except that the exposure to excess EDTA during lysis and sonication was eliminated. Final preparations were stored in 50% glycerol at −80 °C.

All reactions were performed at 25 °C in A25 buffer as described previously (20), except that 0.1 mg/ml bovine serum albumin was not included as standards. Gradients were fractionated into 20 drop fractions (typically 14 full fractions) and analyzed by SDS-polyacrylamide gel electrophoresis with staining by Coomassie Blue. The S₂₀,₀ values of the kinesin head was determined using the least-squares fit of the S₂₀,₀ values of the standards versus their migration. Gel filtration on Sephacryl S-300 (Pharmacia Biotech Inc.) was conducted at ~22 °C in A25 buffer with 25 mM KCl, 0.1 mM MgATP, and bovine serum albumin as an internal marker as described (11). Fractions from centrifugation or chromatography were analyzed by SDS-polyacrylamide gel electrophoresis with staining by Coomassie Blue. For experiments at very low concentration of head domain, the head domain was concentrated from the bulk of each gradient fraction by adsorption to phosphocellulose and elution with SDS sample buffer and then analyzed by polyacrylamide gel electrophoresis with detection by Western blot analysis using SUK4 (22) as the primary antibody as described (23).

Results

Hydrodynamic Characterization—Previous work (11) has established that DKH340 is monomeric, with an S₂₀,₀ value of 3.3 S, whereas DKH392 is dimeric, with an S₂₀,₀ value of 5.2 S, and further work (7, 8, 13, 14) has confirmed and extended this conclusion with other constructs of similar length. To better localize the regions required for dimerization and to determine the influence of dimerization itself, kinesin head domains terminating at a series of positions in the neck region were expressed in Escherichia coli and purified essentially as described previously (11, 20). The velocity of sedimentation during centrifugation in a sucrose gradient was determined over a range of head concentrations in A25 buffer with 25 mM KCl and the S₂₀,₀ values are summarized in Table I. At a concentration of ~1 μM, DKH346, DKH357, and DKH365 have S₂₀,₀ values of 3.5–3.6 S, which are similar to the values for DKH340, which was previously shown to be monomeric (11), whereas DKH381 and DKH405 have S₂₀,₀ values of 5.1–5.7 S, which are similar to the 5.2 S value for dimeric DKH392 (11).

Gel filtration experiments on Sephacryl S-300 (data not shown) indicate that DKH346, DKH357, and DKH365 elute at the same position as DKH340 and thus have similar diffusion coefficients, whereas DKH381 and DKH405 elute at the same position as DKH392. The similarity of both the sedimentation and diffusion coefficients of DKH346, DKH357, and DKH365 to monomeric DKH340 and of DKH381 and DKH405 to dimeric DKH392 indicates that these constructs are also predominantly monomeric and dimeric, respectively, under these conditions as summarized in Table II.

Sedimentation of DKH340–DKH405 at a higher initial head concentration of 20 μM results in increased S₂₀,₀ values (Table I), indicating aggregation to species larger than the dimer. Aggregation is particularly severe for DKH381 and is highly dependent on the salt concentration as indicated in Fig. 1. When 100 or 300 mM NaCl was included, DKH381 sedimented with S₂₀,₀ values of 5.1 and 5.0 S, respectively (Fig. 1, A and B). An S₂₀,₀ value of 5.0 S was also obtained with 1000 mM NaCl (data not shown). These S₂₀,₀ values are consistent with no...
aggregation beyond the dimer at higher ionic strength. With no added NaCl and only 25 mM KCl, DKH381 migrates more rapidly at ~8.0 S (Fig. 1C). There is little DKH381 remaining in the position expected for the dimer, and the peak is asymmetrical, with streaking toward the dimer position. This behavior is consistent with an equilibrium between dimers and higher aggregates that favors aggregates at this high concentration, but is reversible on the time scale of sedimentation. In the complete absence of added salt, DKH381 is largely insoluble at 20 μM and has a visible turbidity that is cleared by brief centrifugation at 13,000 × g with removal of most of the protein. DKH381 remaining in solution sediments at ~9 S (Fig. 1D). Aggregation of DKH381 is also reduced by 120 mM potassium acetate (Fig. 1E), although the value of 5.7 S indicates that dissociation to dimers is not complete. The value of 5.2 S for DKH392 and DKH405 in 120 mM potassium acetate (Table I) indicates that these constructs are not aggregated even at an initial loading of 20 μM. A more detailed analysis of the oligomeric state during ATPase reactions is given below following description of the ATPase properties of the different constructs.

**Stoichiometry of ADP Release on Binding to MTs**—The new head constructs contain tightly bound ADP that can be labeled by incubation with [α-32P]ATP as described previously for native kinesin (24), DKH340 (20), and DKH392 (11). Monomeric DKH340 releases essentially all of its bound ADP on binding to MTs, while dimeric DKH392 releases only half of its ADP (25), and this half-site reactivity of dimeric species is likely involved in generation of the high degree of processivity of kinesin. Analysis of the other head constructs (Table II) indicates that all of the monomeric constructs release ~95% of their ADP on binding to MTs, while all of the dimeric constructs release ~60% of their ADP after 3 s. This release is consistent with the time course reported for DKH340 and DKH392 (25) and indicates that half-site reactivity is restricted to dimeric species.

**ADP Release Rate**—The rate of release of the bound [α-32P]ADP during a chase with excess unlabeled ATP in the absence of MTs (kcat) equals the steady-state ATPase rate for both bovine kinesin (24, 26) and isolated head domain constructs (20, 27, 28). For monomeric DKH340, kcat had previously been shown to be 5-fold higher than the rate of ADP release from dimeric DKH392 (0.025 versus 0.005 s⁻¹) (11, 20) in 25 mM KCl. Analysis of the ADP release rates for all seven head constructs in 25 mM KCl (Table II) indicates that this difference is not due to dimerization because all of the other heads, both monomers and dimers, have low ADP release rates of 0.005–0.010 s⁻¹. The higher rate with DKH340 rather represents an abnormally high basal ADP release rate for DKH340. A similar pattern is observed in 120 mM potassium acetate (Table III), with all of the rates ~2-fold higher.

**MT-stimulated ATPase**—The MT-stimulated ATPase kinetics of the heads in 25 mM KCl were determined as indicated in Table II. All of the heads have high kcat values, but monomers have higher values of 61–96 s⁻¹ compared with dimers at ~40 s⁻¹. In contrast to the relatively similar kcat values, the K0.5(MT) values differ dramatically over almost a 1000-fold range (from 0.02 μM for dimeric DKH381 to 17 μM for monomeric DKH346). All three dimeric constructs have extremely low K0.5(MT) values of ≤0.14 μM when assayed at ~0.01 μM head concentration, whereas the monomeric constructs have much higher K0.5(MT) values of 1.4–17 μM, with the exception of DKH340. The results
of analysis in 120 mM potassium acetate are indicated in Table III, and the pattern is similar, but the $K_{0.5(MT)}$ values are higher due to the higher ionic strength. The $K_{0.5(MT)}$ values for the monomers were not determined because they would be too high to measure in most cases, but the bimolecular rate constant ($k_{cat(ATPase)}$) was still determined from the MT dependence of the ATPase rate at low MT concentrations. The stimulation of the ATPase by MTs exhibits approximately hyperbolic saturation behavior under these conditions, and thus, $k_{cat(ATPase)}$ determined in this way is numerically equivalent to $k_{cat}/K_{0.5(MT)}$.

The $k_{cat}$ for dimeric DKH392 has been shown to be independent of the concentration of DKH392 in the assay over the range of 0.004–0.025 $\mu$M (23) in A25 buffer with 25 mM KCl. This is the maximum range over which the coupled assay using pyruvate kinase and lactate dehydrogenase is readily applicable. This range was extended to 0.5 $\mu$M DKH392 in the absence of pyruvate kinase by determining the initial rate of $P_i$ production using the malachite green method (29). Over the range of 2.5–25 $\mu$M MTs, the ATPase rate with 0.5 $\mu$M DKH392 varied between 36 and 42 s$^{-1}$, with an extrapolated $k_{cat}$ of 41 s$^{-1}$. Thus, there is no significant change in the $k_{cat}$ when assayed at high concentrations of dimeric DKH392.

Oligomeric State of Head Domains in ATPase Assay—The oligomeric state of the constructs during an ATPase assay is influenced by the way in which the assay is conducted. Typically, the assay is initiated by dilution of an intermediate stock of head domain at 0.5–2 $\mu$M into the reaction mixture with spectrophotometric determination of the initial rate over 30–60 s. The final concentration of head domain is typically 0.01–0.03 $\mu$M and was $\sim 0.01$ $\mu$M for Tables II and III. The results of Table I indicate that DKH340–DKH365 have little tendency to aggregate even at 20 $\mu$M, which is $\sim 1000$-fold greater than the concentration in the ATPase assay. Thus, DKH340–DKH365 are predominantly monomeric under the conditions of the ATPase assay. Table I also indicates that DKH381, DKH392, and DKH405 are predominantly dimeric in 120 mM potassium acetate, even at high concentration, and thus will not be aggregated at the lower concentrations of the ATPase assay. The dimers will also not be aggregated at $\sim 0.01$ $\mu$M in ATPase reactions with 25 mM KCl, but some further aggregation may be present in the intermediate stock solution of 0.5–2 $\mu$M, particularly for DKH381.

Several lines of evidence indicate that higher aggregates of DKH381–DKH405 dissociate rapidly to dimers on dilution into the ATPase assay and that the observed ATPase kinetics are due predominantly to dimeric species. If the higher aggregates dissociate slowly and have different kinetics from the dimeric species, then the ATPase properties of aggregates (fraction 6 in Fig. 1C) should differ from those of unaggregated dimers (fraction 10 in Fig. 1A). One potential consequence of dissociation of aggregates over the course of an ATPase reaction is that a lag or a burst would be observed on dilution of fraction 6 into the ATPase assay. The results of Fig. 2, however, indicate that the ATPase reaction of fraction 6 is highly linear. Any lag or burst is either completed within the few seconds required for mixing and response of the coupled assay system or is longer than the $\sim 200$ s for which the reaction remains approximately linear. The possibility that the linear ATPase kinetics of fraction 6 are due to aggregates that do not dissociate over the $\sim 200$ s of total reaction is made unlikely by the fact that the unaggregated fraction 10 of the gradient with 300 mM NaCl (Fig. 1A) exhibits essentially identical linear ATPase kinetics when diluted to the same final concentration (data not shown). Furthermore, analysis of the ATPase of these two gradient fractions over a range of MT concentrations indicates that they have essentially the same $k_{cat}$ and $K_{0.5(MT)}$ values (44.7 ± 0.7 and 31.5 ± 1.8 s$^{-1}$ and 0.032 ± 0.002 and 0.032 ± 0.015 for fraction 6 (Fig. 1C) and fraction 10 (Fig. 1A), respectively). The lower apparent $k_{cat}$ for fraction 10 of the gradient with 300 mM NaCl is expected due to the presence of bovine serum albumin in this fraction (Fig. 1A). These $k_{cat}$ and $K_{0.5(MT)}$ values are similar to those reported in Table II for DKH381 with consideration of the different head concentrations during the assay and different batches of MTs. The similarity of these kinetic properties indicates either that any aggregates present in fraction 6 of the gradient without added NaCl dissociate rapidly to dimers following dilution into the ATPase assay or that the aggregates have the same kinetic properties as unaggregated DKH381 dimers present in fraction 10 of the gradient with 300 mM NaCl. Similar analysis of the peak fractions of the gradients at 20 $\mu$M for DKH381 with 100 mM NaCl or with 120 mM potassium acetate and for DKH392 and DKH405 without added NaCl or with 120 mM potassium acetate indicated that there were no significant differences in $k_{cat}$ or $K_{0.5(MT)}$ values from the results of Tables II and III.

Conversely, there is no evidence for significant dissociation of DKH381–DKH405 to monomers at higher dilution in the ATPase reaction. If dissociation to monomer occurs during the time course of an ATPase reaction and if the rates for the dimer and monomer differ at that MT concentration, then a nonlinear ATPase progress curve would be produced. In particular, the large increase in $K_{0.5(MT)}$ for monomers versus dimers should produce a significant decrease in ATPase rate at low MT concentration on dissociation of a dimer to a monomer. Dimers at low MT levels do not, however, show a major loss of activity with time at high dilution during an ATPase reaction. When a reaction at 0.0056 $\mu$M DKH381 and 0.01 $\mu$M MTs is followed over a long time, the rate after 1200 s is still 73% of the initial rate. Similar small decreases in ATPase rate with time are observed with monomeric constructs and are possibly due to slow denaturation or adsorption to the cuvette. If the monomer of DKH381 had the same kinetic properties as monomeric DKH365, then the rate would have decreased on dissociation of

| Head construct | $k_{cat}$ | $k_{cat}$ | $K_{0.5(MT)}$ | $k_{cat(ATPase)}$ | $k_{cat(ADP)}$ | $k_{cat(ratio)}$ |
|----------------|----------|----------|--------------|--------------------|----------------|--------------------|
| DKH340         | 0.057    | ND       | 119.0 ± 5.6  | 4.3 ± 0.01         | 27.7            |                    |
| DKH346         | 0.013    | ND       | 1.9 ± 0.09   | 0.58 ± 0.01        | 3.3             |                    |
| DKH357         | 0.015    | ND       | 2.9 ± 0.08   | 0.66 ± 0.04        | 4.4             |                    |
| DKH365         | 0.013    | ND       | 6.8 ± 0.5    | 2.1 ± 0.12         | 3.2             |                    |
| DKH381         | 0.015    | 41.0 ± 1.7| 781.0 ± 49.0| 7.8 ± 0.24         | 100.0           |                    |
| DKH392         | 0.016    | 42.0 ± 2.8| 224.0 ± 16.0 | 3.4 ± 0.27         | 66.0            |                    |
| DKH405         | 0.019    | 44.0 ± 4.1| 115.0 ± 6.0  | 1.9 ± 0.03         | 61.0            |                    |

* ND, not determined.
DKH381 to monomers from the observed initial value of 16 s\(^{-1}\) to 0.48 s\(^{-1}\) based on the \(k_{\text{cat}}\) and \(K_{0.5\text{MT}}\), data of Table II. As only a minor decrease in rate of 27% was observed, it is likely that there is no extensive dissociation to monomer over 1200 s and that the initial rate corresponds exclusively to that of the starting dimeric species.

MT-stimulated ADP Release—The rate of MT stimulation of release of \([\alpha-\text{\textsuperscript{32}P}]\text{ADP}\) (\(k_{\text{bi(ADP)}}\)) was determined in 120 mM potassium acetate for comparison with the \(k_{\text{bi(ADP)}}\) values as indicated in Table III. The maximum \(k_{\text{bi(ADP)}}\) value occurs with DKH381, and progressively decreasing values are observed for both longer and shorter constructs, with the exception of DKH340. For both monomers and dimers, the changes in \(k_{\text{bi(ADP)}}\) parallel the changes in \(k_{\text{bi(ADP)}}\) but at different absolute values. Thus, the \(k_{\text{bi(ratio)}}\) of \(k_{\text{bi(ADP)}}\) is \(-4\) for the three monomers DKH346, DKH357, and DKH365 despite considerable variation in the individual values of \(k_{\text{bi(ADP)}}\). For the dimers DKH381, DKH392, and DKH405, the \(k_{\text{bi(ratio)}}\) values are also similar to each other, but at a higher value of 60–100, while the \(k_{\text{bi(ADP)}}\) values vary by >6-fold.

DISCUSSION

Secondary structure predictions indicate that residues 340 to 390 of \textit{Drosophila} kinesin are highly conserved and are likely to be \(\alpha\)-helical and to form a coiled-coil neck that connects the globular head (motor) domain of residues 1–340 to the hinge in the region of proline 399 (see Figs. 5 and 7 of Ref. 11). DKH340 lacks the neck region between amino acids 340 and 390 and is monomeric, whereas DKH392 is dimeric. Thus, the neck region between amino acids 340 and 392 is necessary for dimerization through what is likely a coiled-coil interaction. The more refined deletion analysis presented here indicates that constructs up to the size of DKH365 remain monomeric, but DKH381 and larger constructs are dimers that remain associated even at the low concentrations used in ATPase assays. The region between amino acids 346 and 365 is highly charged, and this may contribute to its inability to associate tightly enough to effect dimerization by itself. Of the 20 residues in this region, 5 are negatively charged, and 9 are positively charged. In addition, there is a gap in the hydrophobic heptad repeat in this region with a \(d\) position occupied by Glu-355 and an \(a\) position occupied by Asn-359. The region between amino acids 362 and 376 contains hydrophobic residues in the heptad repeat positions, and the requirement of this region for stable dimerization suggests that this hydrophobic interaction makes a major contribution to dimerization.

These results are in general agreement with recent work on other related constructs (7, 8, 13, 14). In particular, Correia et al. (13) reported that similar constructs of \textit{Drosophila} kinesin designated K341 and K366 were monomeric and that K401 was dimeric. This work corrects an early report that K401 was monomeric by scanning electron microscopy (27). The dimeric constructs, particularly DKH381, exhibit an increase in the apparent \(k_{\text{20,cat}}\) value at high concentration, and this is likely due to formation of higher aggregates. Similar aggregation was reported for K401 (13). Under the conditions of the ATPase assay, however, these aggregates are likely dissociated to dimers as indicated by ATPase analysis of aggregated DKH381 in 25 mM KCl (Fig. 2) and by the lack of extensive aggregation in 120 mM potassium acetate at 20 \(\mu M\) DKH381. Some analogous dimeric constructs have been reported to dissociate to monomers at the low concentrations typical of ATPase assays (13, 30). Analysis of DKH381–DKH405 indicates that they remain dimeric at the concentrations at which we performed ATPase reactions and that there is no indication of dissociation at long times. The estimation of dissociation of the dimers to monomers at very low concentration is complicated by a number of technical factors, particularly the tendency for these head constructs to adsorb to surfaces. This problem prevented our application of gel filtration chromatography to analysis of DKH381. Adsorption of K401 to the walls of the cuvette during equilibrium centrifugation was also extensive and prevented analysis by Correia et al. (13) at low concentrations. Their value of 0.037 \(\mu M\) for dissociation of K401 was therefore based on experiments at high initial concentrations (0.5–3 \(\mu M\)), and even this estimation still required a large correction for adsorption. Gradient centrifugation in large diameter tubes is not significantly affected by adsorption, and this method indicates that DKH381 remains predominantly dimerized even at an initial concentration of 0.02 \(\mu M\). The lack of any significant dissociation of DKH381, DKH392, and DKH405 during the initial phase of an ATPase assay is further supported by the gel filtration results with DKH405 at low concentration, the relative linearity observed in the ATPase reaction of DKH381 even at high dilution, and the invariance in \(k_{\text{cat}}\) for DKH392 over a range of 0.004–0.5 \(\mu M\).

The monomeric constructs reported here have higher \(k_{\text{cat}}\) values than the dimeric constructs, but this difference is comparatively modest (61–64 versus 38–44 s\(^{-1}\)), with the exception of DKH340. This decreased \(k_{\text{cat}}\) for dimers relative to monomers is consistent with only one head of a dimer being active at a time due to the half-site reactivity. Other work (30) has indicated that a fusion protein of human kinesin analogous to K401 is dimeric, but with a higher \(K_{d}\) value of 0.7 \(\mu M\) and thus is mainly dissociated at low concentration. Analysis of the ATPase properties of this construct has led to the proposal (30) that dimers should have significantly lower \(k_{\text{cat}}\) values of 10.
s\(^{-1}\) compared with monomers with \(k_{\text{cat}}\) values of \(\sim 55\ s\(^{-1}\)\). Given the extreme variation in the kinetic properties with small changes in length of the neck region, even when the sequence is still wild-type, the influence of a non-wild-type extension in this case is hard to predict. Conclusions based on fusion proteins in this sensitive area may not be applicable to any native constructs. The work presented here establishes that high \(k_{\text{cat}}\) values of \(\sim 40\ s\(^{-1}\)\) are characteristic of native *Drosophila* dimeric constructs. The much lower \(k_{\text{cat}}\) values observed with some other preparations are likely due to factors besides dimerization itself. An additional consideration is that active dimers must have \(k_{\text{cat}}\) values of \(\sim 40\ s\(^{-1}\)\) to produce the velocity of sliding that is observed in single motor motility as discussed previously (25).

That the seven head constructs investigated here should all exhibit low basal rates and high extents of stimulation by MTs is not surprising given that they all have the same catalytic domain. What is surprising is the wide variation in the effectiveness of MTs for producing this stimulation. For example, the addition of only 6 amino acids between DKH340 and DKH346 results in an almost 100-fold change in \(K_{0.5(\text{MT})}\), values (0.176 versus 16.8 \(\mu\)M). Equally striking is the extremely low \(K_{0.5(\text{MT})}\) value of 0.02 \(\mu\)M for DKH381. This \(K_{0.5(\text{MT})}\) value is similar in magnitude to the 0.011 \(\mu\)M concentration of DKH381 that was used in the ATPase measurements. Consequently, even this low \(K_{0.5(\text{MT})}\) value must be considered an overestimate because the free MT level will be less than the total MT level due to depletion of free MTs by binding to DKH381 in this tight binding situation. Thus, the \(K_{0.5(\text{MT})}\) values span a range of \(-1000\)-fold (from <0.02 to 16.8 \(\mu\)M). Clearly, conclusions based on a single construct, or even a limited number of constructs, could give results that may not be characteristic of constructs of even very similar size. For example, which of the constructs DKH340-DKH365 should be used as a “generic” monomer for purposes of comparison with the dimeric constructs? Even within the three dimers, the \(K_{0.5(\text{MT})}\) values differ by 7-fold.

What then are the defining kinetic characteristics that differentiate monomer heads from dimers of heads? Tables II and III indicate that there are two significant discontinuities in kinetic properties that occur at the monomer-dimer transition. One clear difference is that all three dimeric constructs release half of their ADP on binding to a MT, whereas all four monomers release essentially all of their ADP (Table II). In contrast, the \(k_{\text{cat}}\) values for the other constructs, both monomers and dimers, are very similar in each buffer. DKH340 also has the highest \(k_{\text{cat}}\) value for the maximum rate at saturating levels of MTs (Table II) and the highest \(k_{\text{cat} (ADP)}\) value of the monomers (Table III). This may represent an abnormally weak interaction with ADP that results in both an elevated \(k_{\text{cat}}\) value in the absence of MTs and an elevated \(k_{\text{cat}}\) value at saturating MT levels. A general facilitation of ADP release would also result in the increased ability for low concentration of MTs to effect ADP release and be responsible for the very low \(K_{0.5(\text{MT})}\) value and high \(k_{\text{cat} (ADP)}\) value of DKH340 compared with the other monomers. Position 340 occurs at the junction of the minimal motor unit and the predicted \(\alpha\)-helical neck domain. The region on both sides of position 340 is highly conserved in all true kinesins, and it may be that the C-terminal part of the motor domain does not fold properly without part of the neck region. Energy-coupled ATPases are unique in that their ATPase cycles have check points through which they do not rapidly proceed unless some coupled event such as interaction with a MT can occur. Incorrect folding of part of the structure such as may occur in DKH340 could accelerate net ATPase cycling if it selectively decreased the constraint that prevents uncoupled ADP release. Another factor is that ADP release may be linked to conformational changes in the neck region that are energetically unfavorable. The absence of the neck domain in DKH340 would facilitate ADP release if this unfavorable conformational change could not occur because there was no neck.

**Acknowledgments—**We thank J. Scholey for SUK4 cells; J. Nagey, S. Adinaira, B. Cobb, and T.-G. Huang for participation in preliminary phases of this work; and the Drug Synthesis and Chemistry Branch of the National Cancer Institute for Taxol.

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