Kar3 is a minus-end directed microtubule motor involved in meiosis and mitosis in *Saccharomyces cerevisiae*. Unlike *Drosophila* Ncd, the other well characterized minus-end directed motor that is a homodimer, Kar3 is a heterodimer with a single motor domain and either the associated polypeptides Cik1 or Vik1. Our mechanistic studies with Ncd showed that both motor domains were required for ATP-dependent motor domain detachment from the microtubule. We have initiated a series of experiments to compare the mechanistic requirements for Kar3 motility in direct comparison to Ncd. The results presented here show that the single motor domain of Kar3 (Met383-Lys729) exhibits characteristics similar to monomeric Ncd. The microtubule-activated steady-state ATPase cycle of Kar3 ($k_{cat} = 0.5 \text{s}^{-1}$) is limited by ADP release ($0.4 \text{s}^{-1}$). Like monomeric Ncd, Kar3 does not readily detach from the microtubule with the addition of MgATP. These results show that the single motor domain of Kar3 is not sufficient for ATP-dependent microtubule dissociation, suggesting that structural elements outside of the catalytic core are required for the cyclic interactions with the microtubule for force generation.

Kar3 is a microtubule-activated ATPase of the kinesin superfamily involved in spindle assembly and integrity in the yeast *Saccharomyces cerevisiae* (reviewed in Refs. 1–5). *KAR3* was originally identified in a screen for genes essential for karyogamy, the nuclear fusion event during meiosis in *S. cerevisiae* (6). In the absence of Kar3, meiosis will not occur because of the failure to proceed beyond prophase. Once the gene was cloned and sequenced, its relationship to conventional kinesin was established. Kar3 is classified as a member of the Kin C subfamily because its motor domain is located at the carboxyl terminus of the polypeptide. Further experiments with Kar3 indicated that its cellular localization was dependent upon an nonmotor polypeptide, Cik1 (7, 8). These results suggested a physical interaction between Kar3 and Cik1. Detailed analysis of this interaction showed that these two proteins heterodimerize along their respective $\alpha$-helical coiled-coil domains (9). Concomitant with this study, it was also demonstrated that Kar3 interacted with Vik1, another nonmotor polypeptide $\alpha$-helical in nature, very similar to Cik1 in both amino acid identity and predicted secondary structure (10). The cellular localization and function of Kar3 is dependent upon its associated polypeptide. When Kar3 is in association with Cik1, its localization is in the spindle midzone during mitosis and is thought to maintain and provide stability to the mitotic spindle (10, 11). This function is in contrast to the Kar3-Vik1 complex, which localizes to the spindle pole and is thought to depolymerize cytoplasmic microtubules during mitosis (11–14) as well as provide opposing force to Cin8 and Kip1 (10, 15, 16). The roles of both Cik1 and Vik1 during meiosis I and II are currently being explored (17).

This novel heterodimeric structure of Kar3 with Cik1 produces a molecule with only one motor domain. This oligomer differs from other Kin C subfamily members that are homodimers with two motor domains. At the COOH terminus of both Cik1 and Vik1 is a globular domain that may also interact with the motor domain of Kar3. This heterodimeric Kar3 complex could be analogous to the interactions of light chains and calmodulin with myosin superfamily members. Alternatively, the COOH-terminal globular domain of Cik1 and Vik1 may act to tether the motor domain to the microtubule, and thereby modulate the Kar3 motor domain for its cellular functions.

Monomeric motor domain constructs have been instructive in experiments to define cooperative interactions. Both conventional kinesin monomeric constructs (18–20) and Ncd monomeric constructs (21, 22) have been studied. The kinesin monomeric constructs were observed to promote motility, but it was thought that this motility arose from numerous motors working together in a multiple motor motility assay as opposed to a single motor promoting movement independently. Conversely, no motility was reported with motor domain constructs of Ncd (23). Mackey and Gilbert (21) observed that the Ncd motor domain did not detach from the microtubule following ATP turnover, a stark difference from the dimeric construct of Ncd that dissociated from the microtubule readily upon the addition of ATP. The interpretation of this experiment was that the second motor domain was required to weaken the affinity of the first motor domain for the microtubule (21, 24).

Kar3 functions with only one catalytic domain; therefore, we assume that its mechanism for force generation must differ from that of a dimeric kinesin such as Ncd that exhibits cooperativity between the motor domains. A glutenathione S-transferase-Kar3 construct has been reported to be motile (16.7–33.3 nm/s), but the motility was slower than dimeric Ncd motility (100–230 nm/s) and much slower than conventional kinesin (500–800 nm/s) (23, 25–34). The motor domain of Kar3 has been crystallized (35), and its structure is strikingly similar to both kinesin (36) and Ncd (37). As the first step toward understand-
standing the mechanochemistry of Kar3, we have analyzed the Kar3 motor domain (Met383-Lys729) using kinetic and thermodynamic approaches. These findings are compared directly to the monomeric Ndc construct MC6 (Met383-Lys706), as well as the Ndc dimeric construct MC1 (Leu383-Lys706).

**EXPERIMENTAL PROCEDURES**

**Materials**—The N-methylanthraniloyl derivatives of adenine nucleotides (mantATP and mantADP) were synthesized and characterized as described previously (38–40). Buffer Conditions—the kinetic and equilibrium binding experiments were performed at 25 °C in ATPase buffer (50 mM HEPES, pH 7.2, with KOH, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate, 1 mM diethiothreitol, and 5 mM sucrose).

**Protein Purification**—The Kar3 motor domain was expressed from the plasmid pMWKar3 (41), and this clone was generously provided by Dr. Sharyn Endow, Duke University Medical Center. Kar3 was expressed in *Escherichia coli* strain BL21(DE3)pLysS and purified as described (35) with slight modifications. Microtubules were assembled from soluble tubulin (cold depolymerized and clarified) and stabilized with 20 μM taxol. Kar3 has been noted to depolymerize microtubules in vitro (25). However, we saw no evidence of Kar3-induced depolymerization of the taxol-stabilized microtubules in sedimentation experiments as described under “Microtubule Equilibrium Binding.” The stability of the microtubules was evaluated in the presence and absence of MgATP.

**Steady-state ATPase Kinetics**—Steady-state ATPase measurements were determined by following the hydrolysis of [α-32P]ATP to form [α-32P]Pi as described previously (42). The rate of ATP hydrolysis as a function of microtubule concentration (Fig. 1B) was fit to the quadratic equation,

\[ k_{\text{burst}} = \frac{A_{\text{max}}(\text{ATP})}{K_{\text{ATP}} - (\text{ATP})} \]

where \( k_{\text{burst}} \) is the rate of product formation (s⁻¹), \( A_{\text{max}} \) is the maximum rate constant of pre-steady-state ATP hydrolysis (s⁻¹), and \( K_{\text{ATP}} \) is the dissociation constant (μM).

**Pulse-Chase Experiments**—To investigate the decreased burst amplitude in the rapid quench experiments, pulse-chase experiments were performed. In these experiments, the time course of ATP turnover was measured by chasing the Mt-Kar3-[α-32P]ATP intermediate with a cold MgATP chase (16–250-fold dilution) for 10 s (7–10 half-lives of enzyme turnover, \( k_{\text{cat}} = 0.5 \text{ s}^{-1} \)) and then quenched with 5 μM formic acid. The data were fit to Equation 3.

**Stopped-flow Experiments**—The pre-steady state kinetics of mant-ATP binding, mantADP binding, mantATP release, Kar3 binding to microtubules, and detachment of Kar3 from microtubules were measured using a SF-2001 KinTek stopped-flow instrument at 25 °C in ATPase buffer. For the experiments with the mantATP and mantADP, the fluorescence emission was measured at 450 nm using a 400-nm cutoff wavepass filter with excitation at 360 nm (Hg arc lamp). The mantATP and mantATP binding data in Fig. 4 were fit to the following equation,

\[ k_{\text{obs}} = k_{\text{on}} \cdot \text{mantAXP} + k_{\text{off}} \]

where \( k_{\text{obs}} \) is the constant obtained from the exponential phase of the fluorescence change, \( k_{\text{on}} \) defines the second-order rate constant for mantADP or mantATP binding, and \( k_{\text{off}} \) corresponds to the observed rate constant of mantADP or mantATP release, as determined by the y intercept. The dissociation kinetics of the Mt-Kar3 complex and the kinetics of microtubule association by Kar3 were determined by the change in turbidity monitored at 340 nm. All concentrations reported are final after mixing.

**ADP Equilibrium Binding Experiments**—These experiments were designed to determine the dissociation constant that the Mt-Kar3 complex has for ADP at equilibrium. By incubating the Mt-Kar3 complex with ATP and allowing the complex to come to equilibrium, any ADP still bound to the Mt-Kar3 complex should pellet with the microtubules when subjected to centrifugation. Kar3 at 2 μM was incubated with microtubules (10 μM tubulin) and with varying concentrations of radiolabeled MgATP (0.1–100 μM) for 30 min. The 30-min incubation allowed all radiolabeled ATP to be converted to ADP + Pi, which was confirmed by thin layer chromatography. The reaction mixture was then centrifuged. The supernatant was removed, and the pellet was resuspended in 110 μl of 4 N NaOH, followed by addition of 110 μl of ATPase buffer. The pellets were not rinsed with additional ATPase buffer. Aliquots of the reaction mixture, the supernatant, and the pellet were evaluated using HPLC setup. These experiments were performed to determine the amount of radiolabeled nucleotide that partitioned in the pellet without any Kar3 active sites being present. The amount of nucleotide present in the Mt-Kar3 pellet was corrected for subtracting the concentration of ADP in the Mt control at each ATP concentration. The data were plotted and fit to the quadratic equation,

\[ \text{Mt-KADP} = 0.5 \cdot (K_c + K_d + \text{ADP}) \]

where Mt-KADP is the concentration of ADP that partitioned with the Mt-K complex, \( K_c \) is total Kar3, ADP is the total nucleotide present, and \( K_d \) is the dissociation constant.

**Acid Quench Experiments**—The pre-steady state kinetic experiments to determine the rate constant for ATP hydrolysis were performed with a rapid chemical quench-flow instrument (ROQ-3, KinTek Corp., Austin, TX) at 25 °C in ATPase buffer. Kar3 and taxol-stabilized microtubules were preincubated for 30 min to form the Mt-Kar3 complex and reacted with [α-32P]ATP. The reaction was then quenched with 5 μM formic acid, expelled from the instrument, and aliquots of each reaction were spotted on TLC plates and developed to separate radiolabeled ADP from ATP. The data were fit to the burst equation,

\[ \text{Product} = A[1 - \exp(-k_{\text{burst}}t)] + k_{\text{burst}} \]

where A is the amplitude of the pre-steady state exponential burst phase, which represents the formation of [α-32P]ADP-P on the active site during the first turnover; \( k_{\text{burst}} \) is the rate of the burst phase; \( t \) is time in seconds; and \( k_{\text{burst}} \) is the rate constant of the linear phase (μM product·sec⁻¹) and corresponds to steady-state turnover. The plot of the burst rate versus ATP concentration was fit to the equation,
The partitioning factor $X_1$ represents the fraction of Mt-KATP that proceeds toward ATP hydrolysis with the following equation.

$$X_1 = \frac{k_{-1,2}}{k_{-1,3} + k_{-1,4}}$$  \hspace{1cm} (Eq. 8)

The mantADP release kinetics were modeled with the assumption that mantADP would not rebind to the Mt-Kar3 complex upon release of ADP because of the 1 µM MgATP chase in the microtubule syringe (Fig. 3). However, the equilibrium binding experiments (Fig. 2) indicated that Kar3 bound microtubules weakly, suggesting that there may be reversals at Step 6 in Scheme 1. We tested the hypothesis that upon collision of the Kar3-mantADP intermediate with the microtubule, mantADP release may not occur at each site. Therefore, the equation,

$$\text{MantADP output} = X_1^*([\text{Mt-Karat}]+[\text{K-mantADP}])$$  \hspace{1cm} (Eq. 9)

with the partitioning factor $X_1$ representing the fraction of Mt-Kar3 ADP that proceeds toward release of mantADP to form the Mt-Kar3 intermediate with the following equation.

$$X_1 = \frac{k_{-1,2}}{k_{-1,3} + k_{-1,4}}$$  \hspace{1cm} (Eq. 10)

RESULTS

Scheme 1 is an ATPase mechanism for the Kar3 motor domain based on the observed kinetics presented in this article with refinement by computer simulation (43). We have compared these results with those of Ncd, another well characterized Kin C kinesin that is also involved in spindle assembly and dynamics (21, 22, 24, 38, 44, 45). The constants for Kar3, monomeric Ncd construct MC6, and dimeric Ncd MC1 are presented in Table I for direct comparison.

All steps in Scheme 1 were measured for the Kar3 motor domain except inorganic phosphate release ($k_{-1,3}$). The microtubule association kinetics were measured ($k_{-1,4}$), but the signal to noise ratio made data collection difficult to interpret because of the relatively small mass of the motor domain in comparison to the microtubule. The data are presented in Table I, but represent only an estimation of the rate constant.

**Steady-state ATP Hydrolysis**—In the absence of microtubules, the rate of ATP turnover by Kar3 is 0.004 s⁻¹. For kinesin superfamly members, this rate is dramatically activated by microtubules (18–21, 44, 46, 47), and Kar3 also displays this property (Fig. 1). For the experiments presented in this paper, three preparations of the Kar3 motor domain were used. The steady-state parameters, based on 11 ATPase assays, are as follows: $k_{cat} = 0.49 ± 0.02$ s⁻¹ (0.36–0.59 s⁻¹), $K_{m,ATP} = 12.2 ± 2.8$ µM (4.0–19.2 µM), $K_{1/2,mt} = 6.0 ± 0.7$ µM (3.9–8.7 µM).

**Equilibrium Binding of Kar3 to the Microtubule**—To ascertain the relative affinity of the Kar3 motor domain construct for microtubules, equilibrium binding experiments were employed. These experiments were performed as a function of microtubule concentration and in the absence of added nucleotide or nucleotide analogs. Fig. 2 shows that the Kar3 motor domain partitioned with the microtubules as a function of microtubule concentration with the $K_{d,mt}$ at 0.68 µM tubulin. Fractional binding reached 100%, suggesting the Kar3 was fully active. This dissociation constant is 3-fold weaker than observed for the monomeric construct of Ncd, MC6 at $K_{d,mt} = 0.20$ µM tubulin (21).

**Pre-Steady State Kinetics of MantADP Release from the Mt-Kar3-MantADP Complex**—The Kar3 motor domain was incubated with a fluorescent analog of ADP, mantADP, at a 1:2 ratio to exchange the ADP bound at the active site for mantADP. The Kar3-mantADP complex was then rapidly mixed with microtubules or with microtubules plus 1 mM MgATP-PNP, 1 mM MgADP, or 1 mM MgATP in the stopped-flow instrument (Fig. 3A). Note that the amplitude of the exponential quenching of fluorescence, monitored as a function of time, is greatest in the presence of the MgATP or MgADP chase, implying that the mantADP nucleotide, once released, may rebind the active site of Kar3 in the absence of the chase. These data may indicate that AMP-PNP binds weakly to the active site as observed for Ncd (24). The observed differences in the fluorescence amplitude based on nucleotide present suggest that the Kar3 motor domain remains in a conformation after microtubule-activated ADP release that permits rapid mantADP rebinding. Monomeric MC6 also exhibited this characteristic (21).

The experiment was repeated as a function of microtubule concentration with MgATP to provide a chase and prevent mantADP rebinding to the active site. The fit of these data to a hyperbola (Fig. 3B) yielded the maximum rate constant of mantADP release, $k_{-1,2} = 0.4$ s⁻¹. This rate constant appears to be the rate-limiting step for the Mt-Kar3 ATPase as it is the most similar to the steady-state $k_{cat}$ at 0.5 s⁻¹. Rate-limiting ADP release was also observed for Ncd, another Kin C kinesin (21, 22, 24, 45, 46, 48).

**Pre-Steady State Kinetics of MantADP and MantATP Binding to the Mt-Kar3 Complex**—Our previous work with MC6 suggested that ADP is in a rapid equilibrium on and off the active site of the Mt-MC6 complex (21). In the case of MC6, this partitioning between ADP binding and ATP binding (M-N-ADP ↔ M-N ↔ M-NATP) appeared to limit steady-state turnover for monomeric Ncd. To investigate if this mechanism accounted for the Kar3 motor domain kinetics, as implied by the mantADP release data, we explored the possibility that ADP could rebind the active site of the Mt-Kar3 complex. In this experiment, a pre-formed Mt-Kar3 complex was rapidly mixed in the stopped-flow with varying concentrations of mantADP. The inset of Fig. 4A shows a typical experiment that performed at 25 µM mantADP. The kinetics are biphasic with a rapid fluorescence enhancement as mantADP moves from the hydrophilic buffer into the more hydrophobic active site of the Mt-Kar3 complex. The slow, second phase of fluorescence enhancement is attributed to a population of Kar3 sites with ADP enhancement is attributed to a population of Kar3 sites with ADP.
increased as a function of mantATP concentration with stopped-flow with varying concentrations of mantATP (Fig. 2). The observed rate of the initial fluorescence enhancement increased as a function of mantATP concentration with \( k_{+1} \) = 1.2 \( \mu M^{-1} s^{-1} \). The slow second phase of fluorescence enhancement is attributed to ADP being present on a population of Kar3 active sites; therefore, for these sites, mantATP binding was limited by the rate of ADP release (0.4 \( s^{-1} \)).

**Acid Quench Kinetics of the Mt-Kar3 Complex**—A pre-formed Mt-Kar3 complex (10 \( \mu M \) Kar3, 30 \( \mu M \) tubulin) was rapidly mixed in the rapid quench instrument with varying concentrations of \( [\alpha^{32}P]ATP \), and the time dependence of ATP hydrolysis was determined (Fig. 5). The results show that there was a burst of product formation (ADP-P) at the active site during the first ATP turnover, indicating that the rate-limiting step occurs after the hydrolysis. The linear phase of the data correlated well with the steady-state experiments for Kar3. The burst rates increased as a function of ATP concentration, and the rate constant for ATP hydrolysis (\( k_{-ATP} \)) was 16 \( s^{-1} \) with the \( K_{d,ATP} \) of 319 nm. The \( K_{d,ATP} \) implies that ATP binding was very weak for the Mt-Kar3 complex, a result in agreement with the mantATP binding data.

Examination of the amplitude of the burst phase reveals that at even very high ATP concentrations (800 \( \mu M \) ATP), the amplitude of the burst did not approach the enzyme concentration used in the experiment. The burst amplitude represents formation of the Kar3-ADP-P intermediate; therefore, this constant can be related to the active sites of Kar3 available to bind and hydrolyze ATP during the first turnover. There are several possible explanations for the reduced burst amplitude. The
first is that there is inactive enzyme present. We assume that the vast majority of Kar3 present in these assays is active because of data presented in Fig. 2 as well as active site assays not shown. Second, there could be ADP still bound at the active site, preventing access to the active site for ATP binding. The results presented in Figs. 3A and 4A indicate that mantADP can rebind the active site; therefore this could be one explanation for the decreased burst amplitude. A third explanation could be a slow on-rate associated with ATP binding to the active site of the MtKar3 complex. Fig. 4B shows that the rate of mantATP binding is 1.2 μM⁻¹ s⁻¹. This rate should not limit ATP hydrolysis. Finally, there could be a significant off-rate (k⁻), implying that there is kinetic partitioning where ATP is in a rapid equilibrium on and off the active site before moving forward in the ATPase cycle to ATP hydrolysis.

Acid Quench Pulse-Chase Comparison of the MtKar3 Complex—To investigate the decreased burst amplitude, pulse-chase experiments were pursued (Figs. 6 and 7). In these experiments, any [α-³²P]ATP bound at the active site would proceed forward toward hydrolysis by the addition of an unlabeled ATP chase. ATP weakly bound at the active site would be diluted by the high concentration of unlabeled ATP. The experimental design is such that it will reveal a stable MtKar3-ATP intermediate if one were to exist. Fig. 6 depicts two experiments performed by mixing the MtKar3 complex (5 μM Kar3, 30 μM tubulin) at 10 and 25 μM MgATP. Note that at each ATP concentration, the amplitude of the pulse-chase transient was significantly higher than the acid quench amplitude. These results show that a stable MtKar3-ATP intermediate can be trapped.

This experiment was repeated at higher ATP concentrations, ranging from 10 to 300 μM (Fig. 7). The results show that the first-order rate of the burst is very rapid and saturates at >500 s⁻¹. The fact that the burst rate in the pulse-chase experiments is not linear and saturates at increasing concentrations of ATP indicates that there is a rate-limiting conformational change (k₁₂, Scheme 1) that occurs prior to ATP hydrolysis. The plot of the burst amplitude versus MgATP concentration (data not shown) never reached saturation and exceeded the enzyme concentration, implying that the Kar3 motor domain remains associated with the microtubule for multiple cycles of ATP turnover as observed for monomeric kinesin and Ncd constructs (18–22).
tial burst phase of each transient were plotted as a function of time. The time course was fit to Equation 3.

To determine whether the concentration of ADP partitioning with the Mt upon centrifugation. Fig. 8 shows that the concentration of ADP with 5 mM formic acid or chased with 5 mM MgATP. A, 10 mM [α-32P]MgATP; B, 25 mM [α-32P]MgATP.

ADP Equilibrium Binding to the Mt-Kar3 Complex—To determine whether the Mt-Kar3 complex could sequester ADP at the active site of Kar3, equilibrium experiments using radiolabeled MgATP were employed (Fig. 8). In these experiments, the Mt-Kar3 complex was incubated with [α-32P]ATP for a period of time sufficient for all the ATP to be hydrolyzed. Any [α-32P]ADP still bound at the active site of Kar3 should partition to the microtubule pellet upon centrifugation. Fig. 8 shows that the concentration of ADP partitioning with the Mt-Kar3 complex reached 0.75 μM, implying that 37.5% of the Mt-Kar3 sites (0.75 μM ADP/2 μM Kar3 sites) have ADP tightly bound to them. The $K_{d,ADP}$ for the Mt-Kar3 complex was 1.67 μM, and this experiment provides additional evidence that ADP can remain bound to the Mt-Kar3 complex.

ATP-induced Dissociation Kinetics of the Mt-Kar3 Complex—Our previous analysis of Ncd showed that the monomeric construct, MC6, failed to dissociate from the microtubule upon the addition of MgATP. Rather, additional salt and MgATP were necessary to weaken the interaction of the motor with the microtubule (21). Similarly, it has been proposed that monomeric kinesin constructs cannot easily dissociate from the microtubule at low salt conditions (18–20). There is evidence from the pulse-chase experiments (data not shown) that the Kar3 motor domain hydrolyzes multiple ATP molecules per encounter with the microtubule, implying that the Kar3 motor domain may not readily dissociate from the microtubule. To explore if this were truly the case, we employed the same experiment that was used to determine whether monomeric Ncd dissociated from the microtubule (21).

In this experiment (Fig. 9), a pre-formed Mt-Kar3 complex was reacted with buffer, buffer with 50 mM KCl, and 1 mM MgATP with 50 mM KCl. The 50 mM KCl was in addition to the 50 mM potassium acetate present in the ATPase buffer. The additional salt weakened the affinity of the motor domain for the microtubule and acted to increase the ATP-dependent signal to noise ratio.

Fig. 9B shows the dissociation of Kar3 from the microtubule as a function of ATP and KCl concentrations. Either ATP or 250 mM KCl did lead to a decrease in turbidity, but the amplitude associated with the turbidity signal was small relative to the amplitude of ATP plus KCl dissociation. The dissociation transients at 200 and 250 mM KCl display aberrant exponential curves, suggesting that the high salt was required to drive all Kar3 motors off the microtubule.

We next performed the dissociation experiment as a function of ATP concentration (Fig. 9C) in the presence of 50 mM KCl to obtain a more robust ATP-dependent signal and to avoid the higher concentrations of KCl that produced stopped-flow transients that were not easily interpretable. The apparent rate constant of dissociation was 6.7 s⁻¹ with the $K_{d,ATP}$ at 5.7 μM.

These data (Fig. 9), taken together, imply that MgATP is not sufficient to dissociate the Mt-Kar3 complex; salt must be added to weaken the affinity with the microtubule. For our studies with monomeric Ncd (MC6), we proposed that there was a small population of monomers that can dissociate from the microtubule in the presence of ATP as reflected by the very small change in turbidity that can be detected (21). The addition of salt weakened the interaction of MC6 with the microtubule, creating a turbidity signal with greater amplitude. The results for ATP-induced dissociation for the Kar3 motor domain mirror the kinetics we saw with MC6 (21). Therefore, the hypothesis that a small population of motor molecules is causing a turbidity signal with less amplitude may also be true for the Kar3 motor domain.

**DISCUSSION**

This study presents the ATPase mechanism of a motor domain construct of the naturally occurring monomeric motor, Kar3. In yeast, Kar3 heterodimerizes with either Cik1 or Vlk1.
with 50 mM KCl, or 1 mM MgATP with 50 mM KCl, and turbidity was rapidly mixed in the stopped-flow apparatus with buffer, buffer
the microtubule (57) and acted to increase the signal to noise ratio. The additional salt was used to weaken the motor affinity to
monitored. The additional salt concentrations, after the contribution of KCl and potassium acetate, ranged from 50 to 300 mM.
[(18), 20, 49–51]. This observation implies that there are mechanistic similarities between the Kin C family members that are distinct from other kinesin superfamily members.
The analysis of the Kar3 motor domain revealed that like monomeric MC6 (21), Kar3 when bound to the microtubule did
rebind ADP. This partitioning may affect the overall steady-state rate of ATP turnover. Furthermore, the rapid quench
kinetics show a decreased burst amplitude, indicative of enzyme sites being unavailable for ATP binding and subsequent
turnover. This behavior was seen with the monomeric Ncd construct, although full burst amplitude was achieved at high
ATP concentrations with MC6 (21), unlike Kar3. For Ncd, we proposed that the partner motor domain was required to return
the motor to the conformation more competent to bind ATP and less accessible for ADP binding. The hypothesis we are pre-
rently testing is that for Kar3, the associated polypeptides Cik1 and Vik1 provide this function.
There do appear to be several differences between the mono-
meric Ncd MC6 and the Kar3 motor domain. The fast ATP off-rate (k−1) that was modeled for the pulse-chase and acid-
quench experiments (Figs. 5–7) was not observed for mono-
meric Ncd (21, 22). Why this difference occurs is not readily
apparent, especially given the sequence and structure similarities. The rapid quench burst experiments are very different as
well. MC6 was able to achieve a burst amplitude equal to its
enzyme concentration (21). Even though both Kar3 and MC6
rebind ADP quite readily, the difference between the bursts
may be rooted in the fast off-rate of ATP binding, as well as the
weak Kd,ATP observed for Kar3. Also, MC6 has a 3-fold tighter
affinity for the microtubule (0.2 μM) than does the Kar3 motor
domain (0.67 μM). Experiments to compare directly the disso-
ciation of both motor domains from the microtubule seem to
indicate that MC6 does not release from the microtubule as readily in the presence of salt as does the Kar3 motor domain
(data not shown). This is consistent with the findings presented here and leads to the hypothesis that the Kar3 motor domain is
better able to dissociate from the microtubule than MC6, but
both require something else structurally to detach from the
microtubule for ATP-dependent force generation. For MC6, it is
the other head of the dimer that is necessary for detachment.
For Kar3, it is unclear what is necessary for microtubule disso-
ciation. A key step in the ATPase cycle of any molecular
motor is the detachment from the filament to take a step to the
next binding site on the filament. Monomeric kinesin and Ncd
motors stay attached to the microtubule, yet they continue to
turnover ATP (18–22). However, this ATP turnover is not
coupled to movement as in the case of dimeric kinesin and Ncd.
Because Kar3 is a naturally occurring monomeric motor, one
hypothesis was that the elements necessary for movement
would be contained in the Kar3 motor domain. We have shown
that this is not the case for the Kar3 motor domain. However,
some if not all of the necessary components for movement
must be contained in the GST-Kar3 construct because it generates
microtubule sliding in vitro (25).
Monomeric Kinesins—This study addresses the question of how a monomeric motor can promote motility. Okada and
Hirokawa (52) propose a mechanism for the processive monomeric kinesin Kif1A that involves the use of the carboxyl-
terminal tail of tubulin to allow the motor to essentially swing
to the next microtubule binding site. This mechanism is not
applicable to Kar3 because Kar3 does not contain the K loop
motif necessary for Kif1A motility. Also, Kar3 probably func-

![Figure 9](image_url)

**Fig. 9** ATP-promoted dissociation kinetics of the Mt-Kar3
complex. A, a Mt-Kar3 complex (5 μM Kar3, 6 μM tubulin, 20 μM taxol)
was rapidly mixed in the stopped-flow apparatus with buffer, buffer
with 50 mM KCl, or 1 mM MgATP with 50 mM KCl, and turbidity was
monitored. The inset represents the experiments where the Mt-Kar3 complex
is rapidly mixed in the stopped-flow instrument with, from top to bottom
trace, 5, 8, 10, 25, and 50 μM MgATP. All reactions contained 50 mM
KCl. The data were fit to a double exponential function. The maximum
rate constant of ATP-promoted dissociation (kprom) was 6.7 ± 0.1 s⁻¹ with
the Kprom,ATP = 5.7 ± 0.3 μM.

(9, 10), but in each case, there is only a single motor domain
present. We have used steady-state and pre-steady state kinet-
ic to investigate the turnover of ATP by the motor domain
to determine what is the minimal functional unit of Kar3
necessary for movement. Table I includes the kinetic constants
derived from the experimental data and refined by computer
simulation, and the mechanism is presented in Scheme 1.

**Kar3 and Ncd**—Our initial observations were that the Kar3
motor domain was very similar to the single Ncd motor domain
constructs reported (21, 22). These motor domains are 46%
identical, and their crystal structures are practically superim-
posable (35). The sequence and structural similarity between these
two motor domains makes comparisons between these
two motors more relevant because both are Kin C motors that
promote minus-end directed microtubule motility and both
function in the spindle. As seen with MC6, the steady-state
ATPase was very slow when compared with monomeric Ncd constructs (18, 20, 49–51). This observation implies that there are
mechanistic similarities between the Kin C family members that are distinct from other kinesin superfamily members.

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tions in a cooperative manner with multiple Kar3 motors, either in the spindle itself or the array of microtubules that forms when two yeast nuclei fuse during karyogamy.

Our analysis of Kar3 motor domain emphasizes the need to understand the role of dimerization with either Cik1 or Vik1 for Kar3 function. Why have two separate polypeptide partners? Manning and Snyder (10) have shown that the localization of the Kar3 motor is dependent upon the polypeptide that is bound. Kar3 when bound to Cik1 localizes to the spindle midzone during mitosis and to the microtubule bundle between nuclei during karyogamy. Mitosis in the yeast S. cerevisiae takes place within the nucleus as there is no nuclear membrane breakdown. During karyogamy, some other factor must mask the nuclear localization signals of both Kar3 and Cik1 for the motor to be exported to the cytoplasm. It is not known whether the mechanochemistry of Kar3-Cik1 is differentially regulated for its function during mitosis as compared with meiosis. Also, the role of the Kar3-Vik1 heterodimer is largely unknown.

For Kar3-Cik1 to be sufficient for the role of dimerization with either Cik1 or Vik1 would explain this differential regulation. Why have two separate polypeptide, analogous to myosin and dynein family members. Our results with Kar3 in comparison to cytoplasmic dynein, Ndc, and kinesin, will provide insight to understand the mechanistic diversity that nature has evolved to promote microtubule-based motility.

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