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Nucleotide-free kinesin motor domains reversibly convert to an inactive conformation with characteristics of a molten globule

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ABSTRACT

Nucleotide-free kinesin motor domains from several kinesin families convert reversibly to a refractory conformation that cannot rapidly rebind ADP. In the absence of glycerol, the refractory conformation of *Drosophila* kinesin motor domains is favored by 50-fold with conversion of the active to the refractory species at ~0.052 s^{-1} and reactivating in the presence of ADP at ~0.001 s^{-1}. This reactivation by ADP is due to conformational selection rather than induced fit because ADP is not bound to the refractory species at concentrations of ADP that are sufficient to saturate the rate of reactivation. Glycerol stabilizes the active conformation by reducing the rate of inactivation, while having little effect on the reactivation rate. Circular dichroism indicates a large conformational change occurs on formation of the refractory species. The refractory conformation binds ANS (8-anilino-1-napthalenesulfonic acid) with a large increase in fluorescence, indicating that it has molten globule character. High ANS binding is also observed with the refractory forms of Eg5 (a kinesin-5) and Ncd (a kinesin-14), indicating that a refractory conformation with molten globule characteristics may be a common feature of nucleotide-free kinesin motor domains.

1. Introduction

Kinesins are a large superfamily of proteins that share a common motor domain that catalyzes microtubule-activated ATP hydrolysis [1,2]. Most kinesins use the energy of ATP hydrolysis to produce movement along microtubules (MTs), although some also function to depolymerize MTs. The kinesin-1 family was the first to be discovered and is the most extensively studied. In the absence of MTs, kinesin-1 (hereafter just called kinesin) hydrolyzes ATP and releases Pi rapidly, but can only slowly release ADP. Interaction with MTs induces a conformational change that accelerates ADP release [3,4]. Fast binding of ATP or ADP coupled with slow release as ADP results in very tight net binding of ADP. In fact when native heterotetrameric kinesin was isolated from bovine brain, it was found to have retained an ADP at its active site in spite of ATP not having been included in the solutions used in purification [3]. ADP itself, in the absence of Mg^{2+}, binds only weakly to kinesin and has a fast dissociation rate of ~18 s^{-1} compared to ~0.03 s^{-1} for release of Mg^{2+} from the Mg^{2+} complex of E·MgADP and ~0.005 s^{-1} for concerted release of MgADP [5,6]. In the presence of millimolar Mg^{2+} concentrations, the initial release of Mg^{2+} at 0.03 s^{-1} is usually followed by rapid Mg^{2+} rebinding to E·ADP before release of Mg^{2+}-free ADP can occur. In the presence of excess EDTA, the released Mg^{2+} is trapped by binding to EDTA and cannot rebind to E·ADP to suppress ADP release. Consequently the net binding of ADP is weak in the presence of excess EDTA. Gel filtration or other methods are now sufficient in the presence of excess EDTA to strip kinesin of its bound ADP. With full length bovine kinesin, this nucleotide-free form was able to rapidly rebind ATP with burst kinetics [4].

A striking early observation was that isolated nucleotide-free motor domains of *Drosophila* kinesin prepared in this way were, however, unable to rapidly rebind ADP, although they did slowly regain ADP binding during prolonged incubation with ATP [7]. This result is in contrast to the relatively stable apo-form of full length bovine kinesin discussed above. Ma and Taylor were able to stabilize isolated human motor domains in an active nucleotide-free conformation by use of 20% glycerol and high salt or by rapid removal of released ADP by apyrase [8,9]. Although human nucleotide-free motor domains also undergo reversible conversion to a refractory form, the rate of inactivation with human motor domains is slower than for *Drosophila* motor domains, which allowed for the study of the kinetics of nucleotide binding to active apo-motor domains. Members of the kinesin-5 and kinesin-14

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families have also been shown to produce an inactive apo-form that can slowly reactivate [10–13].

This report demonstrates that even the more labile *Drosophila* kinesin motor domains can be stabilized by high concentrations of glycerol and further presents a detailed characterization of the reversible transition between active and refractory conformations of nucleotide-free motor domains. The refractory conformation has properties consistent with that of a molten globule and this is also true of the refractory conformation of Eg5 and Ncd as examples of kinesin-5 and kinesin-14 families. These properties help to explain why crystallization of nucleotide-free kinesin motor domains has been difficult.

2. Materials and methods

2.1. Protein preparations

General methods for expression of motor domains in *E. coli* and purification were as previously described [14]. All buffers used in protein purification contained ≥0.1 mM MgATP. K346 is a monomeric *Drosophila* motor domain of amino acids 2–346 with an N-terminal extension of GS and a C-terminal extension of GGTS that was obtained by cleavage with Tev protease of a fusion with thioredoxin (modified pET32 plasmid with a linker containing a 6xHis-tag and a Tev protease cleavage site and with the two cysteine residues of thioredoxin converted to serines). The fusion protein was expressed in *E. coli* with induction by isopropyl-1-β-D-thiogalactose and lysed essentially as described [14]. Kinesin was first batch purified by adsorption to phosphocellulose (P11, Markson LabSales) at pH 6.3; resuspended in 20 mM MES/NaOH pH 6.3 with 100 mM NaCl, 2 mM $MgCl_2$ and 2 mM mercaptoethanol; refiltered; and then kinesin was eluted with T20 buffer (20 mM Tris/Cl pH 8, 2 mM $MgCl_2$) with 800 mM NaCl and 2 mM mercaptoethanol. The high salt phosphocellulose filtrate was adsorbed to an Ni-NTA column (Sigma) and eluted with a 0–200 mM imidazole gradient. His-tagged Tev protease [15] was added to the NTA eluent before dialysis against T20 buffer with 1000 mM NaCl and 2 mM $MgCl_2$ to remove imidazole so that the cleavage mixture could be reapplied to an Ni-NTA column. In the absence of imidazole, cleaved motor domains bind weakly to Ni-NTA and were eluted with a 0–20 mM imidazole gradient (the His-tagged thioredoxin and Tev protease require much higher concentrations of imidazole for elution). K357 is the monomeric motor domain of amino acids 2–357 with an N-terminal extension of GS and a C-terminal extension of GGTS. It was cloned as a fusion protein with maltose binding protein (derived from pMal5c with a linker containing a 6xHis-tag and a Tev protease cleavage site) and was purified and cleaved with Tev protease as described for K346. All the data presented here for *Drosophila* kinesin-1 was obtained with K346 except for the circular dichroism experiments with K357 that undergoes reversible inactivation at similar rates (not shown). K346 and K357 are expected to have similar properties to those of the corresponding non-fusion constructs DKH346 and DKH357 studied previously [16]. H349 is the untagged human Kif5B motor domain consisting of amino acids 1–349 and was purified as previously described [5]. His-tagged human Eg5 motor domain is residues 2–386 [17]. Ncd motor domain was cloned as amino acids 330–700 with N- and C-terminal extensions of MGSSHHHHHHGT and TS respectively. Eg5 and Ncd were purified on phosphocellulose and Ni-NTA essentially as described for K346 except that Ncd was initially adsorbed to P11 at pH 8 and not 6.3.

Because ATP was present throughout the purifications at ≥0.1 mM, these procedures yield the E·ADP complex. Motor domains were stored in small aliquots at −80 °C following dialysis versus A25 buffer (25 mM ACES/KOH (pH 6.9), 2 mM magnesium acetate, 2 mM potassium EGTA, 0.1 mM potassium EDTA) that was supplemented with 50% glycerol, 100–300 mM KCl, 0.1 mM MgATP and 2 mM DTT. Kinesin concentrations were determined by absorption at 280 nm in 6 M guanidine hydrochloride [18]. An extinction coefficient of 2310 $M^{-1}cm^{-1}$ at 280 nm was used for bound ADP in preparations with ADP. Microtubules (MTs) were prepared from porcine tubulin and stabilized with paclitaxel, as previously described [19]. All reactions with MTs contained 10 μM paclitaxel.

Nucleotide-free kinesins were prepared from E·ADP by chromatography in the presence of glycerol and excess EDTA at 4 °C. E·ADP was diluted with 20 mM Mops pH 7.2, 2 mM EDTA, 2 mM DTT and 50% glycerol (v/v); adsorbed to a MacroPrep High S cation exchange column (Bio-Rad); washed extensively with the same 50% glycerol buffer to remove ADP and then eluted with stabilization buffer of 800 mM KCl, 50% glycerol, 20 mM Mops pH 7.2 and 2 mM DTT. All superfamily members prepared by this procedure had negligible MT-ATPase in the absence of added ATP (see Fig. 1B), consistent with essentially complete removal of bound ADP.

2.2. Kinetics analysis

All experiments were conducted at 25 °C in A25 buffer with

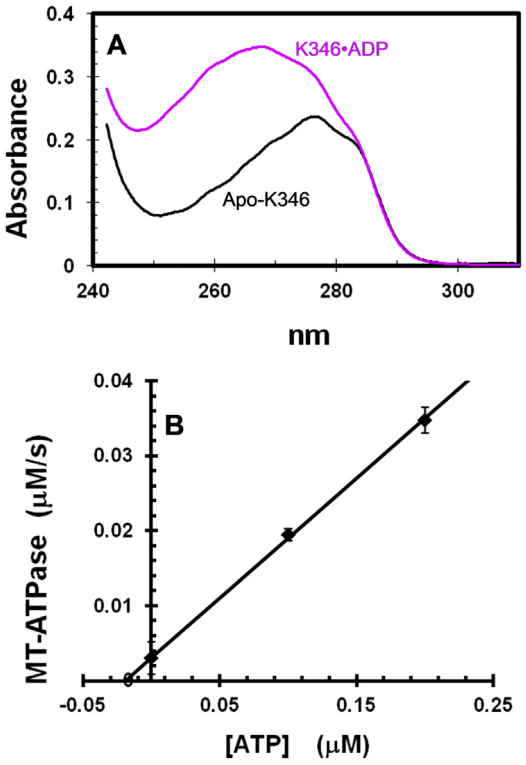

Fig. 1. Evaluation of residual ADP in apo-K346. A. Spectra of K346·ADP and apo-K346 at 15 μM in 6 M guanidine hydrochloride. K346·ADP at >600 μM was dialyzed against buffer with 100 μM free ADP to ensure saturation with ADP and to allow correction for the contribution of free ADP by subtracting the spectrum of an equivalent dilution of the solution outside the dialysis bag. B. MT-ATPase of apo-K346. The reaction was initiated by addition of 0.87 μM apo-K346 to 1.5 μM MTs in an ATPase reaction mixture with 2 mM PEP, 0.2 mM NADH, pyruvate kinase and lactic dehydrogenase, but without ATP. A low ionic strength buffer was used to increase the affinity of kinesin for MTs (20 mM Mops pH7.0, 2 mM $MgCl_2$ and 0.1 mM EDTA). Addition of ATP to 0.1 and 0.2 μM produced a linear increase in rate. Based on this slope, the rate in the absence of added ATP corresponds to that expected for 0.019 μM ATP or 2% of the 0.87 μM K346 sites. This represents an upper limit because the MTs likely contain some free GDP/GTP that would be a substrate for both pyruvate kinase and K346. Note that apo-K346 is not inactivated over time in this assay because it is stabilized by binding to the MTs.