Effect of temperature on kinesin-driven microtubule gliding and kinesin ATPase activity

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Abstract DeCuevas et al. [J. Cell Biol. 116 (1992) 957–965] demonstrated by circular dichroism spectroscopy for the kinesin stalk fragment that shifting temperature from 25 to 30°C caused a conformational transition. To gain insight into functional consequences of such a transition, we studied the temperature dependence of a full-length kinesin by measuring both the velocity of microtubule gliding across kinesin-coated surfaces and microtubule-promoted kinesin ATPase activity in solution. The corresponding Arrhenius plots revealed distinct breaks at 27°C, corroborating the temperature-dependent conformational transition for a motility-competent full-length kinesin. Microtubules were found to glide up to 45°C; at higher temperatures, kinesin was irreversibly damaged.

Key words: Microtubule; Kinesin; Motility; ATPase activity; Temperature; Thermal stability

1. Introduction

Kinesin is a microtubule-associated motor protein that converts chemical energy released by ATP hydrolysis into mechanical energy [1,2]. Its ATPase activity is substantially enhanced by microtubule binding [3,4]. Numerous kinesin isoforms and related proteins, sharing a common motor domain of 340–350 amino acids from the N-terminus [5,6], have been described in various eukaryotic organisms. The most prominent member of the kinesin superfamily is the conventional kinesin, which moves to the plus end of microtubules and contributes essentially to anterograde vesicle transport in neuronal cells [7].

Conventional kinesin represents a heterotetramer consisting of two heavy (120–130 kDa) and two light chains (60–70 kDa) [3,8,9]. Each heavy chain is composed of an N-terminal globular motor domain with both a microtubule binding site and an ATPase-active center, a stalk region, which is responsible for heavy chain dimerization, and a C-terminal globular tail domain, implicated presumably together with the stalk in cargo binding.

In the presence of Mg^{2+} and ATP, microtubules move across kinesin-coated glass surfaces [10], providing the opportunity to imitate anterograde transport processes in vitro. It is known that kinesin-dependent microtubule translocation can be affected by variations of co-factor concentration and physicochemical parameters, including kinesin density, ionic strength, and viscosity [1,11–15].

Studying the stalk fragment of kinesin between 4 and 80°C by means of circular dichroism spectroscopy, DeCuevas et al. [16] revealed two major transitions (at 25–30°C and 45–50°C), indicating alterations in the α-helical structure. To answer the question whether such changes might be functionally reflected, we used a full-length kinesin to study the temperature dependence of microtubule gliding across kinesin-coated glass surfaces and compared the results with the corresponding ATPase activities, measured in solution.

Furthermore, in vitro motility experiments were usually performed at room temperature. Up to now, no data on kinesin-driven motility at temperatures above 30°C have been available. As cellular events in warm-blooded animals proceed at temperatures between 36°C and 40°C, our data obtained with brain kinesin do not only contribute to understand molecular mechanisms of force generation in general, but also proved insight into the functional behavior of kinesin at the physiological temperature of mammals and into its functional stability under conditions of hyperthermia.

2. Materials and methods

2.1. Kinesin preparation

Kinesin was purified from porcine brain homogenates by a combined procedure of ion exchange chromatography, tripolyphosphate-mediated microtubule affinity binding, and gel filtration [3]. The purified kinesin was frozen and stored in liquid nitrogen.

2.2. Microtubule preparation

Microtubule protein was isolated from porcine brain by two cycles of temperature-dependent disassembly/reassembly [17]. The reassembly steps were performed in the presence of 4 M glycerol to reduce the amount of microtubule-associated proteins, co-purifying with tubulin [18]. The residual microtubule-associated proteins were removed by phosphocellulose ion exchange chromatography [19]. Microtubules were formed by 20-min incubation of phosphocellulose-purified tubulin (1 mg/ml) at 37°C in the presence of 1 mM GTP and 10 μM taxol as assembly promoter.

2.3. Microtubule gliding

Taxol-stabilized microtubules, kinesin, and motility buffer (50 mM imidazole, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM dithiothreitol, pH 6.8), supplemented with 100 mM NaCl, 10 μM taxol, and 0.5 mM disodium ATP, were mixed at room temperature, resulting in final concentrations of 40 μg/ml tubulin and 70 μg/ml kinesin. After 10-min preincubation at room temperature, 10-μl drops of this mixture were transferred onto glass slides pretreated with 5 mg/ml bovine serum albumin (BSA), covered with a 18 mm × 18 mm coverslip, and sealed with a mixture of Vaseline, lanolin, and paraffin. The gliding microtubules were visualized by video-enhanced differential interference contrast microscopy using the image processing system Argus 20 (Hamamatsu). Image processing was performed following instructions

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of Weiss and Maile [20]. The gliding velocities were measured using Argus 20 software. To calculate the arithmetic mean and S.D., the data of at least 25 individual microtubules were involved. For plotting the results, Microcal™ Origin™, version 6.0, was used.

2.4. Temperature adjustment

Due to the requirement of an oil immersion objective to visualize microtubule gliding, it is difficult to maintain a constant sample temperature. Warming or cooling of the microscope table alone usually results in a high temperature gradient within the sample. Under these conditions, it was impossible to keep temperature on an exact level throughout the gliding measurement. The problem was solved by warming or cooling both the whole microscope and the sample in a closed chamber, allowing temperature adjustment within the range from 5°C to 45°C. In this way, disturbing heat flow from the objective to the sample (or vice versa) was widely avoided. Sample temperature was controlled by a thermo-couple adjusted in a distance of 5 mm from the observation field.

2.5. ATPase measurement

ATPase activity was measured by a thin-layer chromatographic procedure using radioactively labelled ATP as substrate.

Kinesin (70 μg/ml) was incubated for 60 min (at 5–20°C) or 30 min (at 25–45°C) with taxol-stabilized microtubules (40 μg/ml tubulin) in motility buffer containing 100 μM sodium ATP and 2% (v/v) [α-^{32}P]-ATP (10 μCi/μl, Amersham). Thereafter, 1 μl of each sample was spotted to PEI cellulose F plates (20 cm×20 cm, Merck). Separation of the released [^{32}P]ADP from non-hydrolyzed [^{32}P]ATP was performed in 0.75 M NaH_2PO_4. After drying, the plates were contacted with a phosphor screen in an exposure cassette and kept there overnight. The amount of released [^{32}P]ADP was measured by an optical scanner, type Storm 860, using the ImageQuaNT™ software (Molecular Dynamics). Control measurements were performed without kinesin. The control values were subtracted from those of the samples with kinesin. The ATPase activities given represent the mean values, calculated from the measurement of three parallel chromatographic runs of the same sample.

2.6. Ligand blotting

Drops of kinesin (5 μl, 80 μg/ml) were transferred to nitrocellulose strips (5 mm×80 mm). After blocking with BSA, the strips were incubated for 15 min with taxol-stabilized microtubules (0.8 mg/ml tubulin) in the presence of 1 mM tripolyphosphate. Non-bound microtubules were removed by washing with buffer containing taxol and tripolyphosphate. To stabilize kinesin microtubule binding, the strips were treated for 15 min with 0.1% glutardialdehyde in phosphate-buffered saline (PBS), pH 7.2. After washing with PBS, containing Tween 20 (0.5%) and BSA, and blocking free aldehyde groups with 0.1% sodium borohydride, the strips were sequentially incubated with monoclonal anti-β-tubulin antibody (Boehringer), biotin-labelled anti-mouse anti-IgG (Sigma), and streptavidin-peroxidase conjugate (Sigma). Microtubule binding became visible after development in a staining solution composed of 0.6 ml 4-chloro-1-naphthol (3.0 mg/ml, dissolved in methanol), 10 ml 0.01 M Tris buffer, pH 7.6, and 4 μl H_2O_2 (30%). Control strips were not incubated with either microtubules or the anti-tubulin antibody.

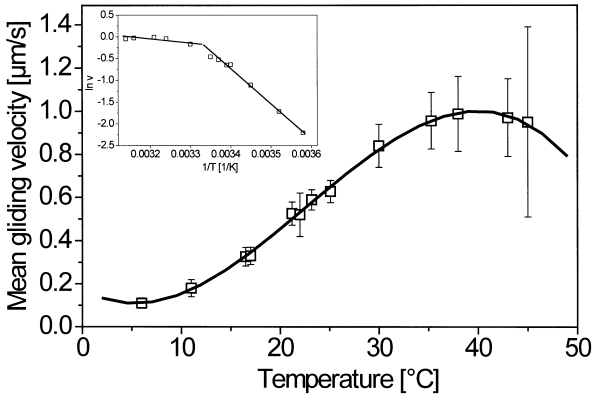

Fig. 1. Dependence of microtubule gliding velocity on temperature. The data were fitted to a polynomial function: $v = 0.18 - 0.027\ t + 0.0029\ t^2 - 0.000043\ t^3$. Inset: Corresponding Arrhenius plot. Linear fitting revealed two distinct intervals, described by the equations: $\ln v = 26.1 - 7891/T$; $r = -0.9949$ for the range from 5 to 25°C and $\ln v = 3.4 - 1088/T$; $r = -0.6182$ for the range from 30 to 45°C. Both linear intervals intersect at 27°C. v: mean gliding velocity; t: temperature in °C; T: temperature in Kelvin; r: regression coefficient.

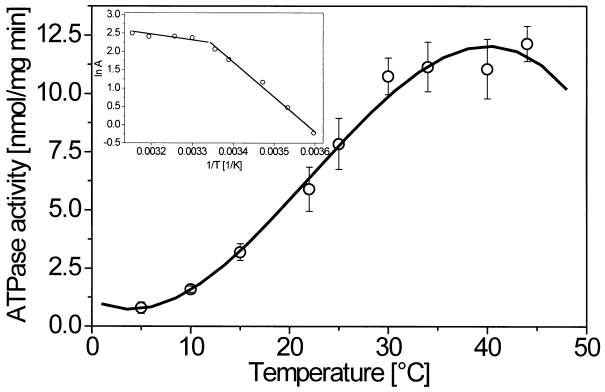

Fig. 2. Dependence of microtubule-stimulated ATPase activity on temperature. The data were fitted to a polynomial function: $A = 1.163 - 0.227\ t + 0.0316\ t^2 - 0.000478\ t^3$. Inset: Corresponding Arrhenius plot. Linear fitting revealed two distinct intervals, which were described by the equations: $\ln A = 33.9 - 9473/T$; $r = -0.9976$ for the range from 5 to 25°C and $\ln A = 4.5 - 645/T$; $r = -0.8323$ for the range from 30 to 45°C. Both linear intervals intersect at 27°C. A: mean ATPase activity; t: temperature in °C: T: temperature in Kelvin; r: regression coefficient.

3. Results

In accordance with the Arrhenius law on temperature dependence of chemical reactions, raising the temperature from 5°C to 37°C resulted in a permanent increase of the velocity of microtubule gliding (Fig. 1). However, the corresponding Arrhenius plot revealed a conspicuous break at 27°C (Fig. 1, inset). The resulting two linear intervals have significantly different slopes, yielding activation energies of 65 kJ/mol for $t < 27$°C, and 9 kJ/mol for $t > 27$°C.

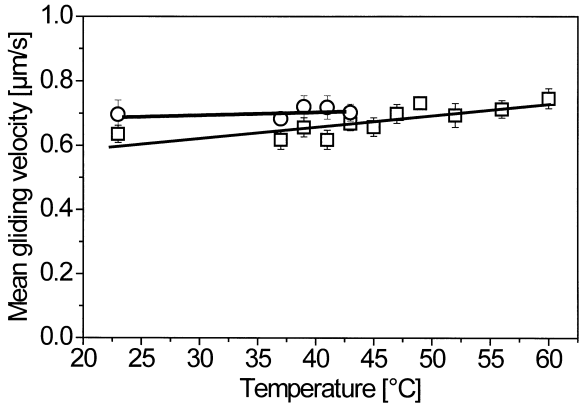

Fig. 3. Microtubule gliding velocities after preincubation of either the microtubules or the kinesin at high temperature. The taxol-stabilized microtubules (squares) or the kinesin (circles) were preincubated for 15 min at temperatures indicated on the abscissa and then used for the motility measurement at room temperature.

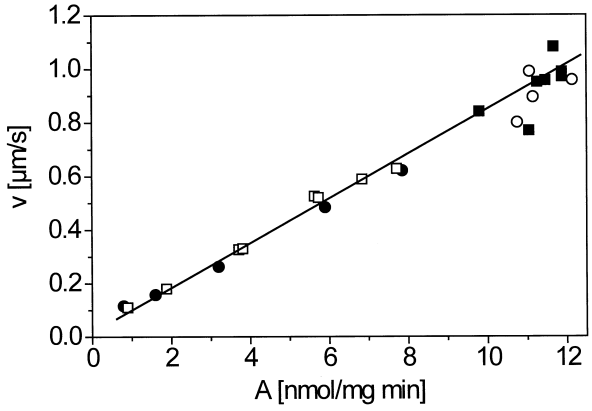

Fig. 4. Correlation between gliding velocity (v) and ATPase activity (A). Using the formula in Fig. 2, the corresponding ATPase activities at given temperature were calculated for the gliding velocities plotted in Fig. 1 (for $t<27$°C open squares, for $t>27$°C solid squares). Using the formula in Fig. 1, the corresponding gliding velocities were calculated at given temperature for the ATPase activities plotted in Fig. 2 (for $t<27$°C closed circles, for $t>27$°C open circles). All data points obtained for A and v might be assigned to the linear function $v=k\ A$, where $k=0.08$ µm s^{-1}/nmol mg^{-1} min^{-1} (regression coefficient $r=0.98767$, $n=24$).

The temperature dependence of gliding velocity corresponded very closely to that of kinesin ATPase activity measured in solution in the presence of microtubules (Fig. 2). The Arrhenius plot of ATPase activity also showed the break at 27°C (Fig. 2, inset). In this case, activation energies of 79 and 5 kJ/mol were calculated for $t<27$ and $t>27$°C, respectively.

From Eadie–Hofstee plots of the dependence of mean gliding velocity on ATP concentration (gliding velocity vs. quotient of gliding velocity and ATP concentration), K_m values of 66 ± 10 µM and 79 ± 8 µM were determined at 25°C and 35°C, respectively. The ATPase measurements yielded K_m values of 29 µM and 32 µM, respectively.

Microtubules were able to glide even at 40–45°C (Fig. 1). However, there was an enhanced tendency of microtubules to detach from kinesin-coated glass surfaces. Consequently, measurements within this temperature interval were difficult, which was expressed by a high standard deviation (Fig. 1). At 45°C, only a few microtubules were still observed to glide; most of them had been released from the surface.

Surprisingly, taxol-stabilized microtubules preincubated at temperatures up to 60°C were able to move across kinesin-coated surfaces at room temperature with the same velocity as non-preincubated ones (Fig. 3). The motor protein was much more sensitive to high temperature. Like in the case of performing the gliding experiments at 39–43°C, the microtubules were found to detach more and more from the glass surface after kinesin pretreatment at corresponding temperature. Preincubation of kinesin at ≥45°C resulted in a total inability of the microtubules to bind to the kinesin-coated surface.

Ligand blots demonstrated that kinesin bound a significantly lower amount of microtubules after 15-min preincubation at 45–50°C than non-preincubated one (not illustrated). Disturbance of kinesin–microtubule interaction was additionally proved by a lowered microtubule-dependent stimulation of kinesin ATPase. At 25°C ATPase activity was 16-fold higher in the presence of microtubules than in their absence; at 45–50°C stimulation was only 2–3-fold.

4. Discussion

Circular dichroism spectroscopy revealed temperature-induced transitions in the kinesin stalk domain between 25–30°C and 45–50°C [16]. To gain insight into the functional consequences of these transitions, we investigated the temperature dependence of both kinesin-dependent microtubule gliding and kinesin ATPase activity.

As is to be expected from the Arrhenius law on temperature dependence of chemical reactions, both the velocity of microtubule gliding and kinesin ATPase activity increased steadily up to about 37°C. However, the corresponding Arrhenius plots revealed distinct breaks at 27°C. Studying ATP binding to myosin subfragment 1, Biosca et al. [21] concluded that breaks in the Arrhenius plots result from alterations in protein conformation. Therefore, our data might be considered the first functional evidence for a change of brain kinesin conformation at 27°C. This temperature-dependent conformational transition seems not to be accompanied by a changed substrate affinity, as the K_m values for ATP at 25°C and 35°C did not differ significantly.

The structural transition observed for the *Drosophila* kinesin stalk fragment between 25°C and 30°C was ascribed to partial melting of the α-helical coiled coil structure within sequence positions 448–595 [16]. As the kinesin heavy chains from mammalian brain and *Drosophila* appear to be highly homologous [22], our results suggest that partial melting of the N-terminal part of the kinesin stalk α-helix results in lowering activation energy.

By elimination of temperature from the plots for gliding velocity (v) and ATPase activity (A), a linear correlation between v and A became evident (Fig. 4). Unlike the Arrhenius plots for gliding and ATPase activity, this plot has a constant slope suggesting a comparable efficiency of transformation of chemical energy into mechanical force for both kinesin conformations. A linear correlation between gliding velocity and ATPase activity is not only a feature of conventional kinesin, but was described also for the *Xenopus laevis* slow kinesin homologue motor protein Eg5 [23].

Microtubules were able to glide even at 40–45°C, significantly exceeding the physiological temperature of mammalian organisms. However, there was an enhanced tendency of microtubules to detach from glass surfaces at $t\geq40$°C. Similar observations were made in motility assays with the *Drosophila* reverse-directed ncd homologue. In this case, however, the number of microtubules attached to the surface decreased at a much lower temperature (30°C) [16]. With the *Xenopus* Eg5, microtubules moved in curved tracks at $t>30$°C, curled up or performed a fish-tailing motion, obviously as a consequence of encountering inactive motor molecules [23]. This indicates that mammalian brain kinesin has a much broader temperature activity interval than kinesin homologue motors of lower organisms.

Detachment of microtubules from the glass might be due to alterations of the motor protein and/or the microtubules. By preincubation of either the kinesin or the microtubules for 15 min at $t\geq40$°C followed by motility measurements at room temperature, we were able to show that mainly changes in kinesin and not in microtubule structure were responsible for the disturbance in microtubule gliding at high temperature. The question arises whether the microtubule and/or the cargo (glass) binding sites of kinesin were damaged. DeCuevas

et al. [16] described at 45–50°C a second conformational change for the kinesin sequence at residues 594–863, containing the carboxy-terminal half of the stalk and additionally 33 residues from the tail domain. As the kinesin tail is believed to bind the cargo, a conformational change in this sequence might explain the disturbed microtubule binding to the glass surface observed in our gliding experiments at high temperature. On the other hand, both the ligand blotting experiments and the remarkably lowered microtubule-based stimulation of kinesin ATPase activity indicate that also the microtubule binding domain of kinesin was damaged at high temperature.

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