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Thermal Activation of Single Kinesin Molecules With Temperature Pulse Microscopy

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Conventional kinesin is a processive motor protein that keeps "walking" along a microtubule using chemical energy released by ATP hydrolysis. We previously studied the effects of temperature between 15° and 35°C on the moving velocity, force, and processivity of single kinesin molecules using a bead assay [Kawaguchi and Ishiwata, 2000b: *Biochem Biophys Res Commun* 272:895–899]. However, we could not examine the effects of temperature higher than 35°C because of the thermal damage to proteins. Here, using temperature pulse microscopy (TPM) [Kato et al., 1999: *Proc Natl Acad Sci USA* 96:9602–9606], we could examine the temperature dependence of the gliding velocity of single kinesin molecules interacting with a microtubule above 35°C up to 50°C (instantaneously, ~60°C), where the velocity reached 3.68 μm/s, the highest ever reported. The Arrhenius plot showed no breaks between 15° and 50°C with a unique activation energy of about 50 kJ/mol, suggesting that the molecular mechanism of kinesin motility is common over a broad temperature range including physiological temperature. Cell Motil. Cytoskeleton 49:41–47, 2001.

Key words: Arrhenius plot; motor protein; microtubule; temperature effect on kinesin; single molecule assay

INTRODUCTION

Kinesin is a motor protein that is responsible for the transport of various types of cargo along microtubules inside eukaryotic cells [Vale et al., 1985; Howard, 1996; Hirokawa, 1998; Vale, 1999]. The remarkable difference between myosin (except myosin V) and dynein is that a single kinesin molecule is sufficient for movement along a microtubule [Howard et al., 1989; Block et al., 1990; Vale et al., 1996] and nucleotide-dependent switching of single- to double-headed binding has been shown [Kawaguchi and Ishiwata, 2001]. Recently, we have reported the effect of temperature on the gliding velocity, processivity, and force generated by single kinesin molecules interacting with a microtubule between 15° and 35°C using a bead assay [Kawaguchi and Ishiwata, 2000b]. The Arrhenius plot was linear in the temperature range examined and the activation energy was estimated

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as 50 kJ/mol. At temperatures higher than 35°C, however, we could not obtain reliable values for the gliding velocities of beads because of thermal deterioration of motor functions.

In order to investigate the interaction between kinesin and microtubule at higher temperatures including physiological temperature, we have applied temperature pulse microscopy (TPM) described previously [Kato et al., 1999] to the gliding movement of a microtubule on kinesin molecules adhering a glass surface. In our TPM, temperature is elevated spatially and temporarily by illuminating a thin metal layer evaporated on the glass surface with infrared laser beam. The temperature of solution adjacent to the illuminated metal thin layer can be elevated even up to a boiling temperature and a concentric temperature gradient (1~2°C/μm) is created around the metal. When the laser beam is shut off, the heat is dissipated into the surrounding medium within 10 ms, so that it is expected that the protein function can be thermally activated beyond the physiological temperature. A preliminary report of this investigation was presented previously [Kawaguchi and Ishiwata, 2000a].

MATERIALS AND METHODS

Proteins

Bovine kinesin extracted from bovine brain was purified by using specific affinity with microtubule [Kojima et al., 1997] followed by DEAE chromatography and sedimentation. Tubulin was prepared from porcine brain and labeled with a fluorescent dye, tetramethylrhodamine succinimidyl ester (C-1171, Molecular Probes, Eugene, OR) according to Hyman et al. [1991]. Microtubules were prepared by polymerizing 20 μM tubulin (labeling ratio of the dye to tubulin heterodimers, about 1.0) in 80 mM PIPES (piperazine-1,4-bis(2-ethanesulfonic acid), pH 6.8), 1 mM EGTA, 5 mM $MgCl_2$, 1 mM GTP, and 36% glycerol at 37°C for 30 min. Microtubules thus prepared were stabilized with 10 μM taxol (T-1912, Sigma, St. Louis, MO).

Flow Chamber for Microtubule Gliding Assay and TPM

To make a heat source for TPM, aluminum was evaporated on a coverslip according to the following procedure [Kato et al., 1999; Yasuda et al., 2000]. First, a coverslip was cleaned by washing in 0.1 M KOH and then 100% ethanol. The cleaned surface was coated with photoresist (OFPR-800, Tokyo Ohka Kogyo Co. LTD., Kanagawa, Japan). The circular-shaped patterns were printed on the surface by irradiating UV light through a circular-shaped mask (diameter, 10 μm). The resist film was developed with NMD-3 (Tokyo Ohka Kogyo Co. LTD), and the irradiated area was removed. Then, the surface was coated with aluminum by vacuum evaporation. The aluminum protected by remaining resist was removed with exfoliation solution of resist. The coverslips thus obtained were then rinsed with distilled water.

Solution containing either 70 μg/ml (high density) or 50 ng/ml (low density) kinesin molecules was introduced into a flow chamber and incubated for 1 min to allow attachment of kinesin to the glass surface of the chamber. The chamber was washed two times with an assay buffer containing 2 mM $MgCl_2$, 80 mM PIPES (pH 6.8), 1 mM EGTA, and 0.7 mg/ml filtered casein (073-19, Nacalai Tesque, Kyoto, Japan) for 2 min each to coat the glass surface with casein and to remove free kinesin molecules. The solution containing fluorescent microtubules was then introduced into a flow chamber and incubated for 2 min to allow binding to kinesin molecules in the nucleotide-free state. The chamber was then filled with the assay buffer containing 1 mM ATP (127531, Boehringer Mannheim, Mannheim, Germany) and an enzymatic oxygen scavenging system (4.5 mg/ml glucose, 0.22 mg/ml glucose oxidase [G-2133, Sigma], 0.034 mg/ml catalase [C-10, Sigma], 10 mM dithiothreitol [DTT]), and sealed with enamel. Other chemicals were of reagent grade.

Temperature Pulse Microscopy (TPM)

Local heating was achieved by illuminating a thin aluminum layer circularly evaporated on a coverslip, 10 μm in diameter, with an infrared laser beam (1W Nd: YLF laser, 1053–1000p: $\lambda = 1.053$ μm; Amoco Laser, IL). The temperature of the microtubule was estimated from the degree of thermal quenching of the fluorescence of tetramethylrhodamine labeled with microtubules. The fluorescence images were taken with a CCD camera (CCD-72; Dage MTI, IN) connected to an image intensifier (KS1381; Video Scope International, Washington, DC) at the video rate of 30 frames/s. The decay lag of this camera system was within one frame (33 ms). According to Kato et al. [1999], the fluorescence intensity at each pixel of the image of labeled microtubule at T°C was divided by that at 20°C (reference temperature) to yield the intensity ratio (r) [Kinosita et al., 1991]. Room temperature was maintained at 20 ± 1°C. We obtained the relation $r = 1\text{-}\alpha(T\text{-}T_0)$, where α and T_0 were, respectively, $0.017°C^{-1}$ and 20°C (Fig. 1).

RESULTS

Effect of Temperature Pulse on Gliding Velocity of Microtubules

In order to investigate the interaction between kinesin and microtubules at higher than 35°C, we applied

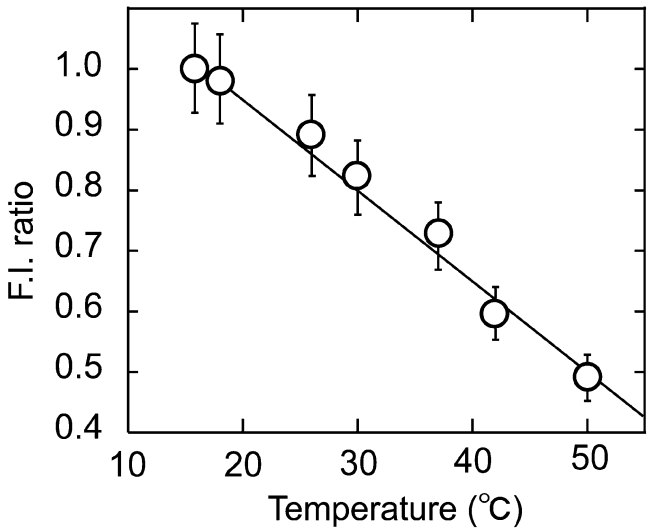

Fig. 1. Relation between fluorescence intensity and temperature. According to Kato et al. [1999], the fluorescence intensity of labeled microtubule at *T*°C was normalized with respect to that at 20°C (reference temperature) to yield the intensity ratio *(r)* in every pixel [Kinosita et al., 1991]. Temperature dependence of *r* was examined on the microscope stage of which temperature was controlled with the use of a thermal insulation chamber, which took about 45 min for stabilization. The reference (room) temperature was maintained at 20 ± 1°C. The data obtained were expressed by the linear relationship, $r=1-\alpha(T-T_0)$, where α and T_0 were, respectively, 0.017°C^{-1} and 20°C.

the TPM [Kato et al., 1999] to the microtubule gliding assay. We examined two densities of kinesin molecules, i.e., high (70 μg/ml) and low (50 ng/ml). At the low density, it was estimated that only one kinesin molecule could interact with each microtubule, such that the pivoting movement of microtubule could be observed [Howard et al., 1989]. The TPM method could thermally activate the motor function beyond the denaturation temperature determined at thermal equilibrium.

First, we examined the effects of temperature gradient on the gliding movement of a long microtubule (10 μm long) at a high density of kinesin. When the temperature of the front portion of a gliding microtubule was higher, the microtubule was straightened and gliding movement appeared to be smooth. However, when the temperature of the rear portion was higher (Fig. 2a), several bucklings were observed at the middle portion of microtubule. This implies that the kinesin motors working at the rear portion produced sufficient force to buckle a portion of microtubule 3μm long (Fig. 2b).

Then, we observed reversible acceleration and deceleration of gliding movement of the microtubule by repetitive application of temperature pulses (every two to several seconds) at high density of kinesin molecules. As shown in Figure 3a, gliding velocities reversibly reached two steady values within 1/30 s after the temperature change: the average velocities were 0.48, 3.65, 0.44, 2.71, 0.33, and 2.24 μm/s, respectively, at 20°, 50°, 22°, 50°, 18°, and 53°C. Here, the local temperature was estimated from the average fluorescence intensity of the gliding microtubule according to the relation, *r(T)*, described above (Fig. 3b). (Note that the coverslip temperature was maintained at 20 ± 1°C.) These data showed that the velocity of the microtubule was gradually decreased by repetitive thermal activation, suggesting that the thermally damaged kinesin molecules may have acted as an internal load for smooth movement of the microtubule. (We could not detect any photo-damage of gliding velocity during observation.) Thus, we considered that only the velocity initially obtained, 0.48 μm/s at 20°C and 3.65 μm/s at 50°C, represented a correct value at each temperature.

Second, we examined the gliding movement at a single molecular level (the low density of kinesin molecules). Before applying temperature pulse, the microtubules showing the pivoting movement were chosen at 20°C. When illuminated with a laser pulse with a duration of about 2 s, an abrupt displacement of the microtubule, by as much as 7.3 μm, occurred, corresponding to the gliding velocity of about 3.68 μm/s (Fig. 4). The average temperature during this 2-s illumination was estimated to be about 50°C. The gliding velocity obtained here (3.68 μm/s at 50°C) is the highest ever reported for single brain kinesin molecules in a normal in vitro motility assay. We confirmed that the gliding velocity obtained by the second temperature pulse was indistinguishable from that obtained by the first one within the experimental error (data not shown), although we succeeded with repetition of temperature pulses on the same *single* kinesin molecules only twice, partly because of the limited length (<10 μm) of microtubules and partly because of the thermal denaturation of proteins during the first temperature pulse. As pointed out by Howard et al. [1989], we found that the velocity was independent of the density of kinesin molecules, even at 50°C (compare 3.65 μm/s in Fig. 3 and 3.68 μm/s in Fig. 4).

Figure 5 summarizes the temperature dependence of gliding velocity of microtubules in the single-molecular assay between 15° and 50°C together with that in the multi-molecular assay between 40° and 50°C. The data shown by the filled circles in Figure 5 between 15° and 35°C were taken from those previously obtained by a single-molecular bead assay [Kawaguchi and Ishiwata, 2000b]. Interestingly, the result was represented by a single exponential curve against the inverse of absolute temperature (Fig. 5a), such that there were no breaks between 15° and 50°C with a unique activation energy of about 50 kJ/mol (Fig. 5b).

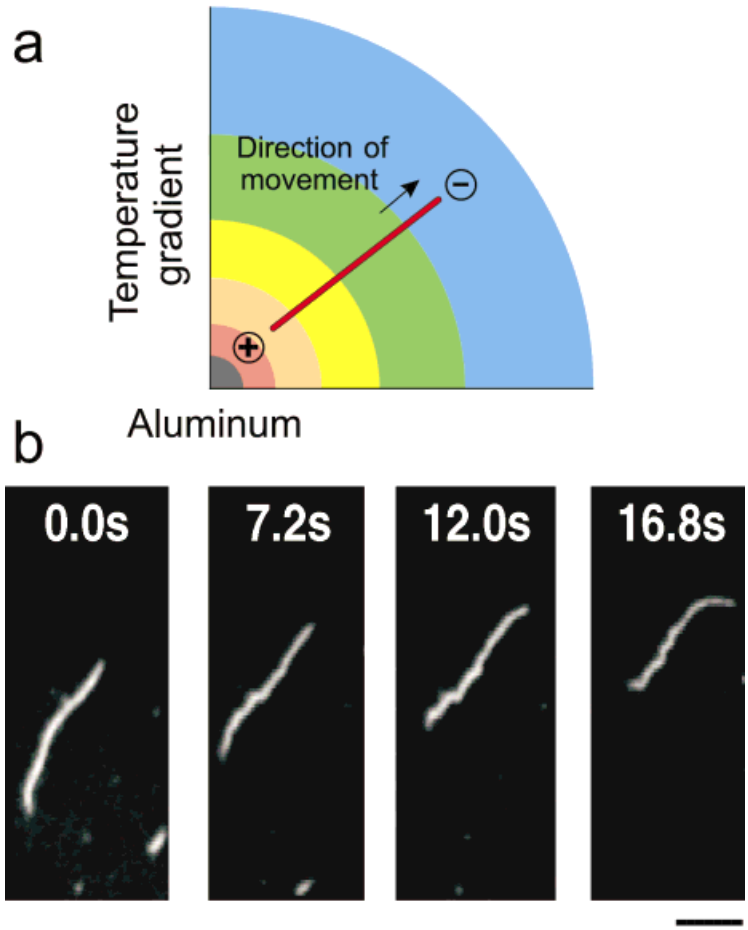

Fig. 2. Imaging of gliding microtubule under temperature gradient created by TPM. **a:** Schematic illustration of TPM system. The circularly aluminum-evaporated surface (10 μm in diameter) was illuminated by focused infrared laser light used for optical tweezers, so that the aluminum became a local heat source, creating concentric temperature gradient around the circle. Gliding movement of microtubule occurred at velocities depending on environmental temperatures. **b:** Effects of temperature gradient on a gliding microtubule (10 μm long). For this experiment, the glass cover was coated with 70 μg/ml kinesin, implying that this experiment was a multi-molecular assay. Bar = 5 μm.

DISCUSSION

Thermal Activation of Kinesins Using TPM

It has been known that the gliding velocity of microtubules interacting with kinesin molecules increases with an increase in temperature [Mazümdar and Cross, 1998; Böhm et al., 2000]. However, this property had not yet been examined by single molecular experiments until recently. In our previous study [Kawaguchi and Ishiwata, 2000b], we examined the temperature effect on single kinesin molecules using a single-molecular bead assay under an optical microscope between 15° and 35°C. In that report, we showed that the gliding velocity increased with the Arrhenius activation energy of 50 kJ/mol, consistent with the temperature dependence of ATPase activity, whereas the generated force was independent of temperature in the above temperature range, 7.34 ± 0.33 pN (n = 70) (Note that this was also the case in the actomyosin complexes) [Kawai et al., 2000]. However, we could not obtain reliable data above 35°C, probably because kinesin was denatured by incubation for 1 min, which was required for obtaining steady temperature in an insulation chamber.

In order to investigate the interaction between kinesin and microtubules at higher temperatures, especially around 40°C (physiological temperature), we applied the TPM described previously [Kato et al., 1999]. In our TPM, it is expected that the motor functions can be thermally activated even at temperatures higher than the denaturation temperature at equilibrium if the duration of the thermal activation is short enough. The gliding velocity thus obtained (Fig. 4) was the highest ever reported for single brain kinesin molecules in a normal in vitro motility assay. It is to be noted that the gliding velocity gradually decreased after each temperature pulse in a multi-molecular assay (Fig. 3), whereas, in a single-molecular assay (Fig. 4), it was kept unchanged, as far as the movement continued. The single-molecular experiments (Fig. 4) suggest that the thermal denaturation of motor function occurs in an all-or-none fashion.

For comparison, we confirmed that thermostatic regulation of the whole stage of microscope at 50°C resulted in the average gliding velocity of microtubules of 2.0 ± 0.1 (n = 7) μm/s at 70 μg/ml kinesin molecules (data not shown), about half of that obtained by TPM

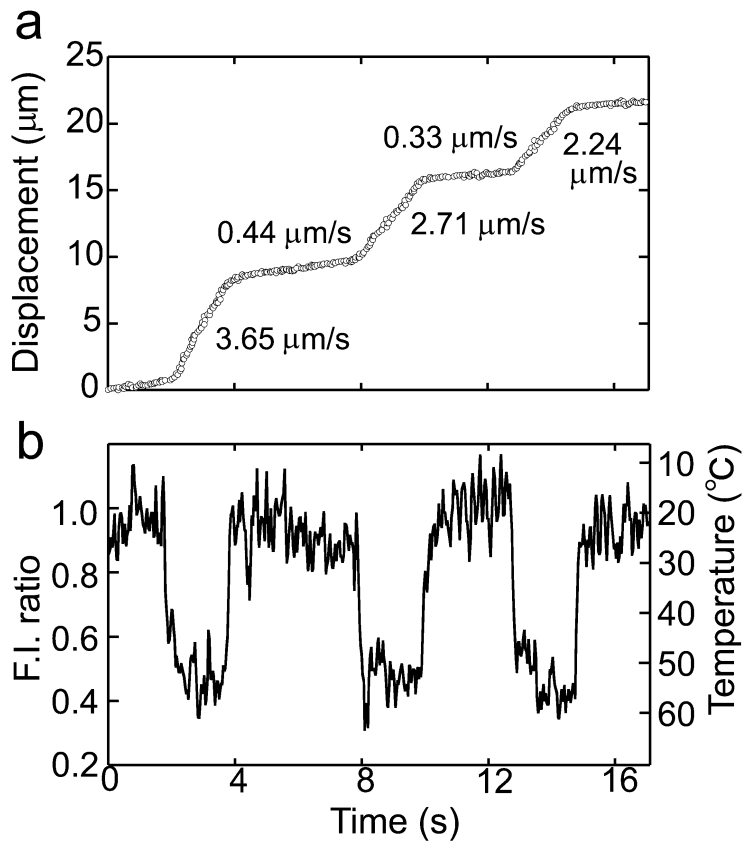

Fig. 3. Time course showing reversible change in the gliding movement of a microtubule with repetitive temperature pulses. **a:** Displacement of the centroid of the fluorescence intensity distribution of microtubule (1.0 μm long) is shown every 1/30 s. **b:** Temperature was estimated by averaging the fluorescence intensity of the image (every 1/30 s) of the microtubule. The temperature of coverslip was kept at 20 ± 1°C. For this experiment, 70 μg/ml kinesin was infused into a cell to perform a multi-molecular assay.

under the same conditions, consistent with the velocity obtained after applying several temperature pulses (see Fig. 3). The gliding motion of microtubules continued at least for 20 min at 50°C in spite of lowered gliding velocity. After incubation for 30 min at 50°C, few gliding microtubules were observed because of the detachment of microtubules from kinesin molecules as reported by Böhm et al. [2000], implying that almost all kinesin (and/or tubulin) molecules were denatured. On the other hand, in an actomyosin motility system, the sliding actin filaments tended to detach from the glass surface, on which heavy meromyosin molecules were adsorbed, within 1 min at 50°C [Kato et al., 1999]. This observation suggests that kinesin-microtubule complexes are more stable than actomyosin complexes against high temperature.

The temperature (T) dependence of gliding velocities (v) from 15° up to 50°C was expressed by a single Arrhenius equation, $v(T) = v_0 exp(-E_a/RT)$, where v_0 is a constant, E_a is an activation energy, and R is the gas constant. In this temperature range, the activation energy turned out to be 50 kJ/mol. Recently, Böhm et al. [2000] reported that the Arrhenius plot of microtubule gliding velocity revealed a break at 27°C, resulting in the activation energies of 65 kJ/mol for $<$ 27°C and 9 kJ/mol for $>$ 27°C. They concluded that the break in the Arrhenius plot results from conformational changes of kinesin and/or microtubule detected by circular dichroism [De Cuevas et al., 1992]. On the contrary, our data showed no break at around 27°C (Fig. 5). As the experiment by Böhm et al. [2000] was not a single-molecular assay but a multi-molecular one, we previously suggested that kinesin molecules that are thermally damaged due to prolonged incubation at high temperatures might have acted as an internal load for the movement of microtubules [Kawaguchi and Ishiwata, 2000b]. This suggestion was confirmed in the present study over the broader temperature range.

In the actomyosin motility system, Kato et al. [1999] reported that the two activation energies, i.e., 100 kJ/mol between 18° and 30°C and 50 kJ/mol between 30° and 45°C, exist in a multi-molecular assay [see also, Kawai et al., 2000]. If the single molecular assay can be performed for actomyosin at various temperatures by using TPM, the unique activation energy might be obtained. Further investigation is needed regarding the actomyosin system.

Temperature Effect on Processivity

In a previous report [Kawaguchi and Ishiwata, 2000b], we showed that the average run length monotonously increased on increasing temperature from 15° to

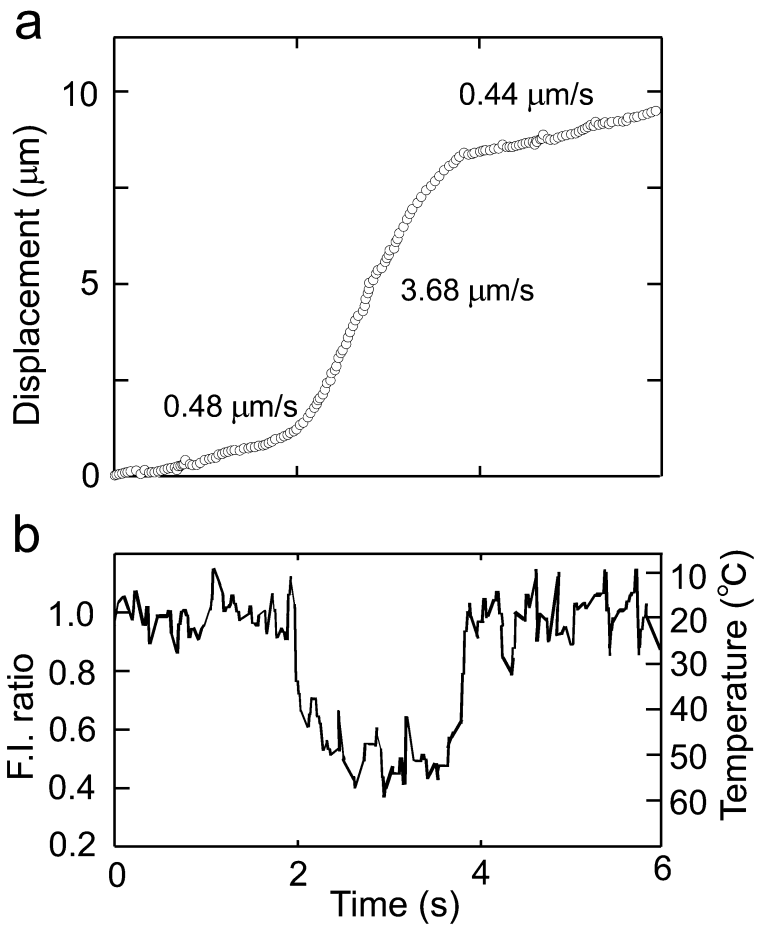

Fig. 4. Time course showing reversible change in the gliding movement of a microtubule on a single kinesin molecule with a single temperature pulse. **a:** Time course of gliding movement of microtubule. Displacement of the tip end of the microtubule (8.4 μm long) is shown every 1/30 s. **b:** Temperature was estimated by averaging the fluorescence intensity of the image (every 1/30 s) of the microtubule. The coverslip temperature was kept at 20 ± 1°C. For this experiment, 50 ng/ml kinesin was infused into a cell to perform a single-molecular assay.

35°C. Although the amount of data was not large enough to confirm the exponential distribution of run length [Block et al., 1990; Vale et al., 1996], we found in the present study that the average run length at around 50°C was approximately 9 ± 2 μm (e.g., Fig.4), 1.8 times longer than that (5 μm) at 20°C. This temperature dependence of run length is consistent with the previous one obtained in a single-molecular bead assay [Kawaguchi and Ishiwata, 2000b] except that the absolute value of run length obtained by the gliding assay was longer than that obtained by the bead assay. As pointed out by Block et al. [1990], the reason why the run length in the gliding assay (5 μm) [Howard et al., 1989] was longer than that in the bead assay (1.4 μm) at room temperature may be that the diffusion coefficient of a microtubule is smaller than that of a bead, so that rebinding of microtubule with kinesin preferably occurs in the gliding assay, apparently resulting in the longer run length [Kawaguchi and Ishiwata, 2000b].

The run length is considered to be proportional to the number of consecutive steps of kinesin along a microtubule. On increasing temperature, the rate limiting step(s) in the ATPase cycle is accelerated, so that the gliding velocity (stepping rate) coupled with the ATPase activity is also accelerated. Then, why was the ratio of run length at 20°C and 50°C only two, in contrast to seven in the gliding velocity (see Fig. 5a)? The run length must be determined by the stepping rate times the lifetime of attached state (probably, the lifetime of a single-headed binding state) of kinesin on the microtubule. Thus, the reason why the temperature dependence of run length is relatively small may be that the lifetime of the attached state decreases on increasing temperature. The temperature dependence of the lifetime of each nucleotide state of kinesin interacting with a microtubule, which is either in the single- or double-headed binding state, must be examined in the future.

CONCLUSION

Using temperature pulse microscopy (TPM), we have examined the gliding velocity at between 15° and 50°C of microtubules interacting with single or multiple kinesin molecules. The corresponding Arrhenius plot showed no breaks, with a unique activation energy (50

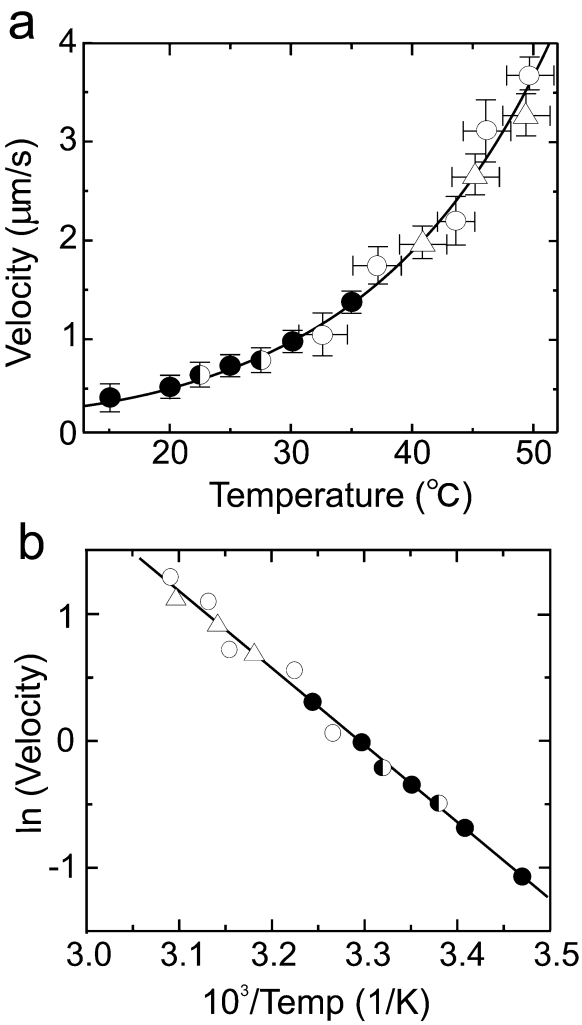

Fig. 5. Temperature dependence of gliding velocity of kinesin molecules. **a:** Gliding velocity vs. temperature. The data from 15° to 35°C (*closed circles*) were taken from the results of single kinesin-coated beads reported previously [Kawaguchi and Ishiwata, 2000b]. The data shown by *half-filled circles* at 22.5° and 27.5°C were taken from those obtained by using a temperature insulation chamber as reported previously [Kawaguchi and Ishiwata, 2000b], and from the pivoting movement of microtubules on single kinesin molecules. The data above 32.5°C were taken from the present results obtained by the single-molecular (*open circles*) and the multi-molecular (*triangles*) assays. The data obtained by the multi-molecular assay were taken only from those at the first temperature pulse because the velocity gradually decreased upon repetitive temperature pulses (Fig. 3). The error bars are standard deviations obtained from 4–6 data. b: Arrhenius plot of a. The error bars are omitted.

kJ/mol), suggesting a common molecular mechanism of kinesin motility over the broad temperature range.

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REFFERENCES

Block SM, Goldstein LSB, Schnapp BJ. 1990. Bead movement by single kinesin molecules studied with optical tweezers. Nature (Lond) 348:348–352.

Böhm KJ, Stracke R, Baum M, Zieren M, Unger E. 2000. Effect of temperature on kinesin-driven microtubule gliding and kinesin ATPase activity. FEBS Lett 466:59–62.

De Cuevas M, Tao T, Goldstein LSB. 1992. Evidence that the stalk of *Drosophila* kinesin heavy chain is an α-helical coiled coil. J Cell Biol 116:957–965.

Hirokawa N. 1998. Kinesin and dynein superfamily proteins and mechanism of organelle transport. Science 279:519–526.

Howard J. 1996. The movement of kinesin along microtubules. Annu Rev Physiol 58:703–729.

Howard J, Hudspeth AJ, Vale RD. 1989. Movement of microtubules by single kinesin molecules. Nature (Lond) 342:154–158.

Hyman A, Drechsel D, Kellogg D, Salser S, Sawin K, Steffen P, Wordeman L, Mitchison T. 1991. Preparation of modified tubulins. Methods Enzymol 196:478–485.

Kato H, Nishizaka T, Iga T, Kinosita KJr, Ishiwata S. 1999. Imaging of thermal activation of actomyosin motors. Proc Natl Acad Sci USA 96:9602–9606.

Kawai M, Kawaguchi K, Saito M, Ishiwata S. 2000. Temperature change does not affect force between single actin filaments and HMM from rabbit muscles. Biophys J 78:3112–3119.

Kawaguchi K, Ishiwata S. 2000a. Effect of temperature on force generation and velocity of single kinesin molecule. Biophys J 78:122A (Abstract).

Kawaguchi K, Ishiwata S. 2000b. Temperature dependence of force, velocity and processivity of single kinesin molecules. Biochem Biophys Res Commun 272:895–899.

Kawaguchi K, Ishiwata S. 2001. Nucleotide-dependent single- to double-headed binding of kinesin. Science 291:667–669.

Kinosita K Jr, Ito H, Ishiwata S, Hirano K, Nishizaka T, Hayakawa T. 1991. Dual-view microscopy with a single camera: real-time imaging of molecular orientations and calcium. J Cell Biol 115:67–73.

Kojima H, Muto E, Higuchi H, Yanagida T. 1997. Mechanics of single kinesin molecules measured by optical trapping nanometry. Biophys J 73:2012–2022.

Mazümdar M, Cross RA. 1998. Engineering a lever into the kinesin neck. J Biol Chem 273:29352–29359.

Vale RD. 1999. Kinesin, conventional. In: Kreis TE, Vale RD, editors. Guidebook to the cytoskeletal and motor proteins, 2nd ed. Oxford: Oxford University Press. p 398–402.

Vale RD, Reese TS, Sheetz MP. 1985. Identification of a novel force-generation protein, kinesin, involved in microtubule-based motility. Cell 42:39–50.

Vale RD, Funatsu T, Pierce DW, Romberg L, Harada Y, Yanagida T. 1996. Direct observation of single kinesin molecules moving along microtubules. Nature (Lond) 380:451–453.

Yasuda K, Okano K, Ishiwata S. 2000. Focal extraction of surface-bound DNA from a microchip using photo-thermal denaturation. Biotechniques 28:1006–1011.