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Mutual sensitization of ATP and GTP in driving F-actin on the surface-fixed H-meromyosin

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Abstract

Using an in vitro motility assay on acto-H-meromyosin, we studied the sliding velocity of an actin filament driven by two kinds of H-meromyosin heads: H-meromyosin head with ATP bound (fast motor) and with GTP bound (slow motor). We found a significant increase in the sliding velocity owing to the coexistence of the fast motor and the slow motor. This phenomenon may give an important suggestion with regard to the integration over multiple interactions of H-meromyosin heads along the actin filament.

Keywords: Acto-H-meromyosin; Sliding velocity; In vitro motility assay; ATP hydrolysis; GTP hydrolysis

1. Introduction

Contraction of muscle is brought about by a cyclic interaction between myosin heads and actin filaments with concomitant hydrolysis of ATP on the myosin heads. To investigate quantitatively the underlying molecular mechanism of actomyosin motility, a novel method of in vitro study using a fluorescence microscope was developed [1–9]. Under the microscope, a few micrometers of fluorescently labeled actin filament moved smoothly and continuously. The actin filament interacted with approximately 140 myosin heads per 1 μm of actin filament [7], indicating that the observed sliding velocity of the actin filament is an integrated quantity of the multiple interactions of the actin filament with neighboring myosin heads.

In order to study the mechanism of the integration, we measured the sliding velocity of actin filaments, one of which was interacting with two kinds of heavy meromyosin (H-meromyosin) head simultaneously, namely, a H-meromyosin head with ATP bound and one with GTP bound. GTP is a non-physiological substrate of H-meromyosin. In preliminary work, we found that H-meromyosin heads with GTP bound drove actin filaments at a very low speed (slow motor), and that the velocity of actin filament in 15 μM ATP increased from 0.60 to 0.74 μm/s upon addition of 2 mM GTP [10]. H-meromyosin heads with GTP bound did not impede the sliding of the actin filament driven by H-meromyosin heads

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with ATP bound (fast motor), but rather prompted it. This observation was very striking because the result was difficult to conform to the traditional idea where the sliding velocity of the actin filament is determined by the total balance between the positive forces (exerted from fast motors) and the negative forces (exerted from slow motors) [3,11–13]. In order to get more insight into this phenomena, a systematic study was made in the present study under the conditions of various molar ratios of ATP and GTP in the driving solution.

2. Materials and methods

2.1. Preparation of muscle proteins

Actin was extracted from an acetone-dried powder of rabbit skeletal muscle and purified according to a previous method [14] with a slight modification [15].

Myosin from rabbit skeletal muscle was obtained according to the method of Perry [16] with a slight modification. Myosin dissolved in a solution containing 10 mM PIPES (pH 7.0), 500 mM KCl, 1 mM DTT was subdivided into plastic micro test tubes dipped in liquid nitrogen to be frozen rapidly and then stored at −80°C [8]. H-meromyosin was prepared by α-chymotryptic digestion of myosin [17,18] and used within a day for the in vitro motility assay.

2.2. Motility assay of actin filaments labeled with rhodamine phalloidin

2.2.1. Flow cell

Coverslips and slide glasses for fluorescence microscopy were cleaned in KOH/ethanol and then rinsed with distilled water and finally air dried [2,5]. A few drops of 0.1% nitrocellulose dissolved in isoamyl acetate were placed onto the coverslip to form a nitrocellulose film on the surface [17]. The excess nitrocellulose solution on the coverslips was removed by air drying. Two pieces of spacer were mounted on the slide glass with silicone vacuum grease. The coverslip coated with nitrocellulose film was fixed onto the spacers on the slide glass by silicone vacuum grease. The coverslip, two pieces of spacer and slide glass were further fixed by VALAP (vaseline/lanolin/paraffin (1:1:3(w/w/w))) to avoid accidental movement of the coverslip during operation of the objective lens. The volume inside the flow cell was approximately 35–40 μl.

2.2.2. Measurement of the sliding velocity of actin filaments

The sliding velocity of the actin filament was measured according to the method described in detail previously [8,9,17]. F-actin dissolved in the solution containing 25 mM KCl, 4 mM $MgCl_2$, 0.1 mM EGTA and 25 mM imidazole (pH 7.4 adjusted with hydrochloride at 28°C), was mixed with 1.3-fold molar excess of rhodamine phalloidin, kept on ice overnight and used within two weeks. The procedures of the in vitro motility assay were as follows: 80 μl of H-meromyosin solution (60–80 μg/ml) in MB solution containing 25 mM KCl, 4 mM $MgCl_2$, 0.1 mM EGTA, 25 mM imidazole (pH 7.4 adjusted with hydrochloride at 28°C) and 1% 2-mercaptoethanol, was introduced into the flow cell. After 60 s, the H-meromyosin solution was replaced with 80 μl of MB solution containing 2 mg/ml bovine serum albumin (BSA) to block the nitrocellulose surface where H-meromyosin was not bound and to wash out this unbound H-meromyosin. After 60 s, 40 μl of 11 nM F-actin labeled with rhodamine phalloidin in MB solution containing 0.5 mg/ml BSA was introduced so as to bind to the immobilized H-meromyosin. After 60 s, the flow cell was washed out with 80 μl of MB solution containing 0.5 mg/ml BSA. Next, the solution was replaced with AB solution containing 24 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 0.1 mM EGTA, 0.5mg/ml BSA, 25mM imidazole (pH 7.4 adjusted with hydrochloride at 28°C), 1% 2-mercaptoethanol and an oxygen scavenger system (0.1 mg/ml glucose-oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose) which was preincubated at 28°C. The flow cell was set up on the microscope stage. The image of the actin filaments was brought into focus under the microscope. Then, 80 μl of either SB solution containing 24 mM Na^+, 10 mM K^+, $[MgCl_2] = [GTP] + 4$ mM, 0.1 mM EGTA, 0.5 mg/ml BSA, 25 mM imidazole (pH 7.4 adjusted with hydrochloride at 28°C), 1% 2-mercaptoethanol, oxygen scavenger system, ATP-regenerating system and various nucleotide(s), or SBL solution containing 12 mM Na^+, 6 mM K^+, 16 mM

Li^+, $[MgCl_2] = [GTP] + 4$ mM, 0.1 mM EGTA, 0.5 mg/ml BSA, 25 mM imidazole (pH 7.4 adjusted with hydrochloride at 28°C), 1% 2-mercaptoethanol, oxygen scavenger system and various nucleotide(s), was preincubated at 28°C and was introduced into the flow cell. The ATP-regenerating system contained 20 units/ml pyruvate kinase and 1 mM phosphoenolpyruvate. Pyruvate kinase is a relatively nonspecific enzyme and can regenerate GTP from GDP [19]. To adjust the ionic strength of SB solution by the addition of GTP, the amount of Na^+ and K^+ was kept constant after the addition of NaCl and KCl upon consideration of Na^+ and K^+ from GTP stock solution (pH 7.0). The ionic strength of SB solution was approximately 51 mM at no GTP, 52 mM at 1 mM GTP, 53 mM at 2 mM GTP, 55 mM at 4 mM GTP and 59 mM at 8 mM GTP in the absence of ATP. The remarkable increase in sliding velocity in 1 mM ATP was not observed between 50 mM and 60 mM [20]. Similarly, the amount of Na^+, K^+ and Li^+ in SBL solution was kept constant. SB solution at the GTP assays and SBL solution at the GTP analogs assays (Fig. 7) were used according to the positive monovalent ions from stock nucleotide solutions (GTP, GTP(γS) and GDP). The temperature of the experiment was 28°C.

The contamination of ATP in GTP from Yamasa Co. was not found within a detectable limitation of HPLC (weight ratio ATP:GTP = 1:1000 [21]). Furthermore, we checked the possible contamination of ATP in GTP preparation according to the modified method of Cohn et al. [22]. We used 10 units/ml hexokinase and 3 mg/ml glucose in order to specifically hydrolyze any contaminating ATP in GTP preparation solutions. Actin movement in 15 μM ATP was completely stopped with the hexokinase treatment.

The fluorescence images of actin filaments recorded on video tapes were retrieved into frame memories in the personal image analyzer system. At the condition of slow actin movement, two or more frames in video rate (1/30 s) were averaged in order to improve the S–N (signal–noise) ratio of the image; the average image was used for the analysis. The number of averaged frames was set so that actin filaments could not move beyond 1 pixel. The image of one frame was used in the case of the fast actin movement (greater than 1 μm/s). In order to measure the centroid position of the actin filament accurately, we selected the filaments that were not transforming at the microscopic level. Firstly, we measured both the maximum intensity of the analyzed filament and the minimum intensity around it. Secondly, we converted the filament image into the thresholding image and confirmed that the filament image corresponded to the outline image. The threshold was set manually at 3/5–4/5 of the sum of the maximum intensity and the minimum intensity. The centroid position of the filament which was obtained was almost independent of the threshold in this range. Finally, we measured the centroid position of the actin filament.

We executed the above procedures for 4 filament images of time interval between 20 and 2/30 s. As a result, we got the total translocation of the centroid position of the actin filament in a pixel unit for the time between 60 and 6/30 s; actin filaments moved more than approximately 25 pixels with a few exceptions. We then calculated the sliding velocity of the actin filament using the following conversion coefficient. The silicon intensifier target camera image was crooked. We divided the image into 400 areas (each area contained 25×25 pixels). For each area, we assigned the conversion coefficient of a pixel unit to micrometer, separately, in the vertical and the horizontal direction; the average coefficient was about 94 pixels per 10 μm. We adopted the filaments whose three speeds for each interval on the way to the analysis of one filament was within ± 0.3 of the average from its total displacement, in reference to the data filtering of Homsher et al. [20]. At least twenty filaments were sampled under each condition. The movement of actin filament was represented as follows: (A) in the presence of the ATP-regenerating system, the mean velocity and the standard error of the mean; (B) in the absence of the ATP-regenerating system, the velocity at $t = 0$ and its standard error.

As the concentration of ATP in the flow cell of the in vitro motility assay was lowered, fragmentation of the actin filament occurred during sliding on the surface-fixed myosin [3,5]. This was also the case even in the presence of the ATP-regenerating system. When actin filament was driven by GTP alone, however, the fragmentation was negligible. Even when a low concentration of ATP was added in

the range of GTP between 1 and 8 mM, the actin filament did not break and continued to slide smoothly. The effect of GTP analogs on the fragmentation of actin filament at low ATP concentration was in the following order: low ATP ≫ + GDP⟩ + GTP(γS)⟩ + GTP > GTP alone.

2.2.3. Equipment

F-actin labeled with rhodamine phalloidin was observed with a fluorescence microscope (Olympus BH-2; Tokyo, Japan) equipped with UVFL X100 objective lens (oil immersion numerical aperture = 1.3), and a 100 W ultra high pressure mercury arc lamp. The microscope stage was equipped with a home-made warm plate to control temperature to within ±0.1°C. The images of actin filaments were taken using a high-sensitivity television camera (Hamamatsu C2400; Hamamatsu, Japan), enhanced by an image processor (Hamamatsu Argus-100; Hamamatsu, Japan) and recorded on SVHS videotapes. The fluorescence images were analyzed with a personal image analyzer system (PIAS LA-500; Osaka, Japan).

2.3. Rate of nucleotide hydrolysis in solution

F-actin used in the measurement of the nucleotide hydrolysis rate of acto-H-meromyosin was prepared as follows: G-actin was polymerized in a solution containing 40 mM KCl, 2 mM $MgCl_2$, 25 mM imidazole (pH 7.4 adjusted with hydrochloride) and then mixed with 1.3-fold molar excess of phalloidin and was kept on ice overnight. The resulting F-actin was washed for at least four cycles of centrifugation and suspension in the solution until excess ATP had been removed as determined by the absorbance at 259 nm in the supernatant [19].

The nucleotide hydrolysis of acto-H-meromyosin was determined in SBP solution containing 24 mM Na^+, 10 mM K^+, $[MgCl_2] = [GTP] + 4$ mM, 0.1 mM EGTA, 25 mM imidazole (pH 7.4 adjusted with hydrochloride at 25°C), 56 units/ml pyruvate kinase, 1 mM phosphoenolpyruvate and nucleotide (15 μM ATP, 0.1–2 mM GTP). The activity of the ATP-regenerating system was sufficient in comparison with the NTPase of acto-H-meromyosin. At a given time interval, the reaction was stopped with 0.3 M perchloric acid. The amount of inorganic phosphate (Pi) liberated was determined calorimetrically according to the previous method [23]. The hydrolysis rates were obtained from the average slope. Actin concentration was 1.5 mg/ml, and H-meromyosin concentration was 0.023 mg/ml. The temperature of the experiment was 25 ± 0.1°C.

2.4. Chemicals

ATP, GTP(γS), pyruvate kinase and phosphoenolpyruvate were purchased from Boehringer-Mannheim (Tokyo, Japan). Rhodamine phalloidin was from Molecular Probes (Eugene, OR). GTP and GDP were from Yamasa Co. (Chyouchi, Japan). Glucose-oxidase, catalase, glucose, α-chymotrypsin and imidazole were from Sigma Chemicals (Tokyo, Japan).

Protein concentrations were determined using the protein assay method (Bio-Rad laboratories; Tokyo, Japan), biuret method and ultraviolet absorption method: $A^{1\%}_{290nm} = 6.3$ for G-actin [24], and $A^{1\%}_{280nm} = 6.47$ for H-meromyosin [25]. The molecular weights used in the calculation were 42000 for G-actin and 340000 for H-meromyosin.

3. Results

3.1. The sliding velocity of actin filaments on the surface-fixed H-meromyosins with GTP bound

In the presence of 4 mM GTP (without ATP), actin filaments were translocated in the direction of their length smoothly and continuously on the surface-fixed H-meromyosin. The sliding velocity increased with the amount of GTP and saturated at several millimolar GTP (Fig. 1). The maximum sliding velocity at infinite GTP concentration was 0.16 μm/s, which was approximately 40 times smaller than the sliding velocity given by ATP (6.66 μm/s; [20]). The GTP concentration for the half maximum sliding velocity was 2.6 mM.

We checked ATP contamination in GTP solution. (A) Elimination of the possible contamination of ATP in GTP solution; We added 10 units/ml hexokinase and 3 mg/ml glucose to SB solution containing 4 mM GTP. The solution was left standing for 3 min at 28°C in order to hydrolyze any contamination

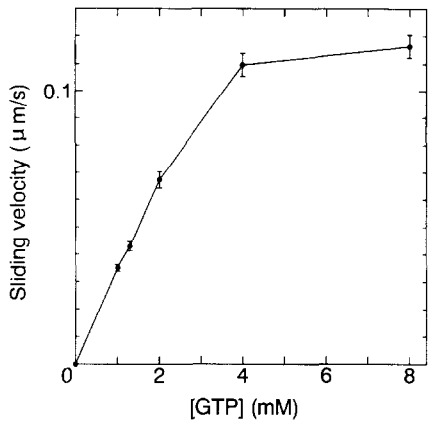

Fig. 1. GTP-dependence of the sliding velocity of the actin filament. The movement of actin filament was observed in SB solutions containing 0–8 mM GTP; temperature 28°C. The solutions contained the ATP-regenerating system. The point is mean velocity and the bar is the standard error (SE) of the mean ($n = 20$–27). The maximum sliding velocity at infinite GTP concentration was 0.16 μm/s. GTP concentration for the half maximum sliding velocity was 2.6 mM.

of ATP. Although the actin filament was left moving by this treatment, the sliding velocity was reduced by about 0.02 μm/s (from 0.110 ± 0.004 μm/s; mean ± SE, $n = 21$). This indicated that the actin filament was really driven by GTP; however, GTP solution may contain a slight contamination of ATP. (B) Estimation of the amount of possible contamination of ATP in GTP solution. In order to estimate the amount of ATP contamination, we measured the effect of addition of 4 μM ATP to 4 mM GTP solution. With the additional 4 μM ATP, the sliding velocity of the actin filament increased to 0.320 ± 0.007 μm/s ($n = 31$). The increment of sliding velocity in the presence of 4 mM GTP was 0.053 μm/s per μM ATP. The reduction of the sliding velocity in (A) corresponded to approximately 0.4 μM ATP. The mutual sensitization described in the latter sections cannot be explained by the possible contamination of ATP in GTP preparation.

The GTPase activity of acto-H-meromyosin in SBP solution was characterized as shown by the open circles in Fig. 2, where GTPase showed a simple hyperbolic dependence on the amount of GTP. A double reciprocal plot of the results gave $V_{max} = 2.7\ s^{-1}$ per head. The GTP concentration where the half maximum velocity of GTPase was obtained was approximately equal to 280 μM. The above results indicated that the H-meromyosin head with GTP bound was a slow motor that hydrolyzed nucleotide at a slow rate and drove actin filament at a low velocity, in contrast with the fast motor of the H-meromyosin head with ATP bound. The filled circles in Fig. 2 show the total rate of nucleotide hydrolysis in the presence of both 15 μM ATP and an increasing amount of GTP. Clearly ATPase of acto-H-meromyosin was inhibited with the increasing amount of coexisting GTP. The dashed line was a theoretical fit obtained under the assumption that the binding of ATP and GTP to the active sites of H-meromyosin was competitive and that the hydrolysis of nucleotides on individual H-meromyosin heads was independent.

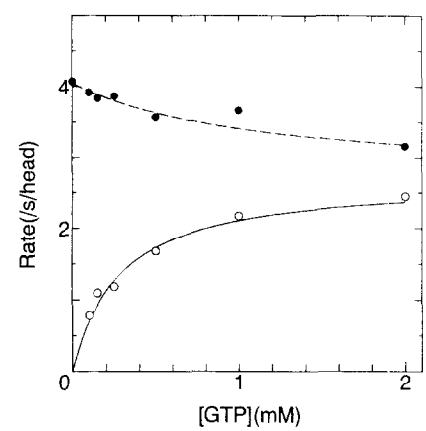

Fig. 2. Dependence of the nucleotide(s) hydrolysis of acto-H-meromyosin both in the absence and in the presence of 15 μM ATP in solution on the varying GTP concentration. The rate of P_i liberation was measured in SBP solutions containing 0.023 mg/ml H-meromyosin, 1.5 mg/ml F-actin and 0.1–2 mM GTP in the absence of ATP (○) and in the presence of 15 μM ATP (●) according to the method of Youngberg and Youngberg [22]. GTPase of acto-H-meromyosin showed a simple hyperbolic dependence on the amount of GTP: $V_{MAX(GTP)} = 2.7\ s^{-1}$ head^{-1}, $K_{m(GTP)} = 280$ μM (solid line). Under the same condition, $V_{MAX(ATP)}$ and $K_{m(ATP)}$ of ATP were 5.4 s^{-1} head^{-1} and 5.0 μM respectively (data not shown). On the basis of assumption that (A) GTP competes with ATP for binding to the enzymatic site in H-meromyosin heads [19] and (B) both ATPase and GTPase obey Michaelis-Menten kinetics independently of each other, the total rate of nucleotide hydrolysis (V) were calculated using the following equation: $V = \{V_{MAX(ATP)}[ATP]_{added} / K_{m(ATP)} + V_{MAX(GTP)}[GTP]_{added} / K_{m(GTP)}\} / \{[ATP]_{added} / K_{m(ATP)} + [GTP]_{added} / K_{m(GTP)} + 1\}$. The dashed line was the theoretical curve at $[ATP]_{added} = 15$ μM.

It is important to note that the GTP concentration for the half maximum sliding velocity (Fig. 1) was much higher than that for the half maximum GTPase activity (Fig. 2) of acto-H-meromyosin in the same way as ATP [3,12]. Here we wish to raise a question of what would happen to the sliding velocity of the actin filament when ATP and GTP are mixed in the driving solution? One may consider that the slow motor of H-meromyosin head with GTP bound will impede actin movement driven by the fast motors of H-meromyosin heads with ATP bound. Results of our preliminary study on this problem showed that, under suitable conditions of the ATP/GTP ratio, the slow motor of H-meromyosin head with GTP bound accelerated the actin filament [10]. To get a much deeper insight into this problem, we made a systematic study, and the results obtained are given in the following sections.

3.2. Mutual sensitization of GTP and ATP in driving actin filaments on the surface-fixed H-meromyosin

3.2.1. The dual-effect of 4 mM GTP

In Fig. 3, ATP-dependence of the sliding velocity of the actin filament both in the presence and absence of 4 mM GTP are shown. GTP affects the sliding velocity of actin filament in a dual way depending on the concentration of ATP. At the high concentrations of ATP (120–500 μM), the sliding velocity decreased with the presence of 4 mM GTP (Fig. 3). For instance, the sliding velocity in 120 μM ATP which was 3.32 ± 0.06 μm/s (mean ± SE, $n = 44$) decreased to 2.78 ± 0.04 μm/s ($n = 28$; 84% of the control) in the presence of 4 mM GTP. Similarly the sliding velocity 4.50 ± 0.06 μm/s ($n = 40$) in 250 μM ATP decreased to 3.78 ± 0.07 μm/s ($n = 27$; 84% of the control). The decrease in velocity was statistically significant ($P < 0.1\%$, student's t-test). These velocity reductions due to 4 mM GTP tended to be negligible at infinite concentration of ATP, suggesting that the reduction of velocity was essentially due to competitive binding of ATP and GTP to active sites of H-meromyosin head (Fig. 3). However, the velocity reduction was less than the reduction of population of H-meromyosin head with ATP bound (that can be expected by apparent binding constants of nucleotides to acto-s1); the population of H-meromyosin head with ATP in the presence of both 250 μM ATP and 4 mM GTP was estimated at 43% of the control of ATP alone [26].

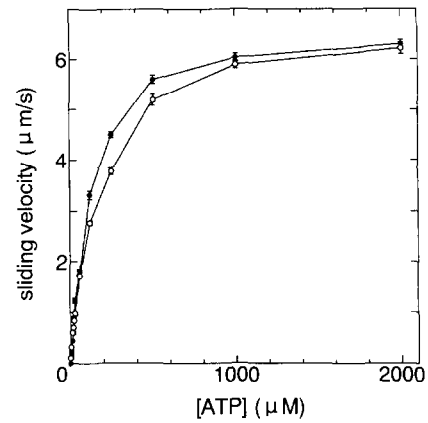

Fig. 3. Effect of varying ATP concentration on the sliding velocity of actin filament both in the absence and in the presence of 4 mM GTP. The movement of actin filament was observed in SB solutions containing 0–2 mM ATP, in the absence of GTP (●) and in the presence of 4 mM GTP (○); temperature 28°C. The solutions contained ATP-regenerating system. The point is the mean velocity and the bar is the standard error of the mean ($n = 25$–52). The maximum sliding velocity at infinite ATP concentration was 6.9 μm/s regardless of the presence of 4mM GTP. ATP concentration for the half maximum sliding velocity in the absence of GTP was 140 μM.

At lower ATP concentrations (Fig. 4), the sliding velocity of actin filament increased in the presence of 4 mM GTP. The sliding velocity in 11 μM ATP which was 0.441 ± 0.005 μm/s (mean ± SE, $n = 30$) increased to 0.603 ± 0.014 μm/s ($n = 26$; 137% of the control) in the presence of 4 mM GTP ($P < 0.1\%$, student's t-test). The acceleration by coexisting GTP was notable. According to the Huxley model [11], the driving force generated by myosin head and the internal resistance of myosin head to actin motion balance at the maximum sliding velocity. If the model can be applied to our experiment, H-meromyosin head with GTP bound (slow motor) will become a net resistance to the faster sliding of actin filament than the maximum velocity of the slow motor (0.16 μm/s), consequently leading to a decrease in the sliding velocity.

3.2.2. The sensitization by GTP of the sliding velocity of actin filaments driven by H-meromyosin heads with bound ATP

To confirm the acceleration effect by coexisting GTP at low ATP concentration, we made a system-

atic study by changing the concentration of both ATP (0–21 μM) and GTP (0–8 mM). Fig. 4 shows the ATP-dependence of the sliding velocity in the presence of several constant amounts of GTP. In order to analyze the effect quantitatively, we introduced a physical parameter, $\delta V = \Delta V/\Delta[\mathrm{ATP}]$, the velocity increment per μM added ATP whose dimension is (μm/s)/(μM ATP). When GTP = 0, the sliding velocity increased almost in proportion to the amount of ATP up to 21 μM. The velocity increment, δV, which was constant at 0.0417 ± 0.0004 μm/s per μM ATP (mean slope ± SE) is just intrinsic to ATP. When GTP coexisted, the velocity increment, δV, became dependent on both ATP and GTP; the velocity increment in the range of ATP between 4 and 11 μM was 0.053 ± 0.001 μm/s per μM ATP (slope ± SE) in 1 mM GTP. This value of velocity increment was larger than that intrinsic to ATP as described above ($P < 0.1\%$), indicating that coexisting GTP enhanced the driving activity of added ATP. The velocity increment, δV, decreased at higher GTP; 0.040 ± 0.002 μm/s per μM ATP in 4 mM GTP, 0.026 ± 0.002 μm/s per μM ATP in 8 mM GTP at the range of ATP between 4 and 11 μM.

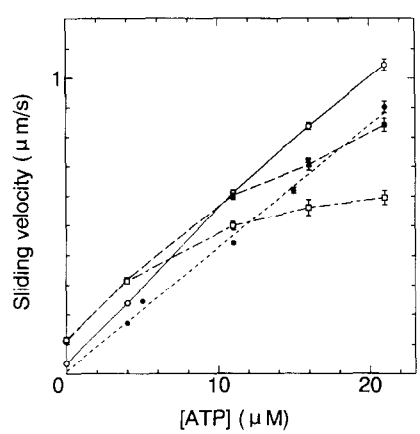

Fig. 4. Effect of varying ATP concentration (0–21 μM) on the sliding velocity of actin filament both in the absence and in the presence of GTP (1, 4, 8 mM). The movement of actin filament was observed in SB solutions containing 0–21 μM ATP plus no GTP (●, · · ·), 1 mM GTP (○, ————), 4 mM GTP (■, — —) or 8 mM GTP (□, - · -); temperature 28°C. The solutions contained ATP-regenerating system. The dotted line was obtained after weighted linear regression of the data in the absence of GTP; the slope was 0.0417 ± 0.0004 μm/s per μM added ATP. The point is the mean velocity and the bar is the standard error of the mean (n = 20–33).

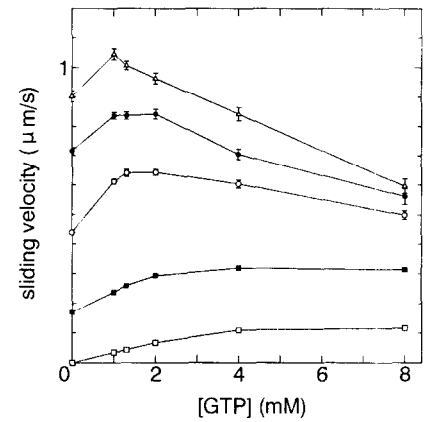

Fig. 5. Effect of varying GTP concentration (0–8 mM) on the sliding velocity of actin filament in the presence of ATP (0–21 μM). The movement of actin filament was observed in SB solutions containing 0–8 mM GTP plus no ATP (□), 4 μM ATP (■), 11 μM ATP (○), 16 μM ATP (●) or 21 μM ATP (△); temperature 28°C. The solutions contained ATP-regenerating system. The point is the mean velocity and the bar is the standard error of the mean (n = 20–33).

3.2.3. The sensitization by ATP of the sliding velocity of actin filament driven by H-meromyosin heads with GTP bound

In Fig. 5, GTP dependence of the sliding velocity of actin filament in the presence of constant amounts of ATP are shown. As the amount of coexisting ATP increased, the GTP concentration, where sliding velocity was maximum, became lower. However, the maxima of the extra sliding velocity (due to GTP addition) were approximately constant (less than 0.2 μm/s). Therefore the velocity increment per mM of added GTP was significantly larger than without ATP (Fig. 5). This indicated that ATP also enhanced the driving activity of added GTP. This mutual sensitization observed between ATP and GTP was thus an indication of cooperation between ATP and GTP in their driving activities.

3.2.4. The time course of the sliding velocity in the presence of 15 μM ATP, and the effect of 2 mM GTP

The sliding velocity of actin filaments in 15 μM ATP without the ATP-regenerating system decreased

gradually with time (slope = -0.027 ± 0.004 μm/s per min, n = 47; dashed line (4) in Fig. 6). Such a deceleration disappeared if the ATP-regenerating system was present (solid line (3)), suggesting that the deceleration was due to ATP consumption. The result was a confirmation of our previous observation [10]. If 2 mM GTP was present, the deceleration in 15 μM ATP disappeared even without the ATP-regenerating system (dashed line (1)). Further addition of the ATP-regenerating system did not affect the velocity (solid line (2)). These results may be interpreted as follows; 2 mM GTP competed with 15 μM ATP for the binding to enzymatic active sites of H-meromyosin heads, and consequently the rate of ATP consumption was reduced [10].

3.3. The effect of GTP(γS) and GDP on the sliding velocity

GTP was hydrolyzed by acto-H-meromyosin, which caused the sliding of actin filament on the surface-fixed H-meromyosin. We examined the effect of GTP analogs. GTP(γS) alone could not support the sliding movement of actin filaments on

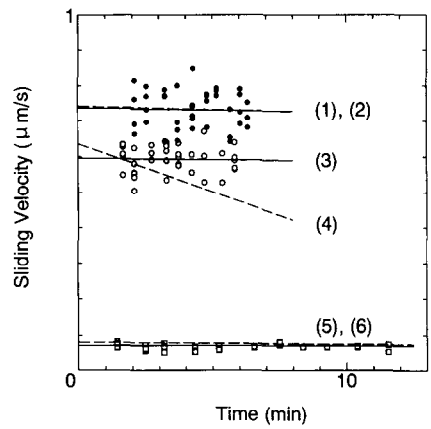

Fig. 6. Time course of the sliding velocity of actin filament. $t = 0$ was the time when SB solution containing nucleotide(s) was introduced into the flow cell. The movement of filaments was observed in SB solutions containing 2 mM GTP (□), 15 μM ATP (○), 15 μM ATP plus 2 mM GTP (●) in the presence of ATP-regenerating system (20 units/ml pyruvate kinase plus 1 mM phosphoenolpyruvate); temperature 28°C. The time average sliding velocity of different filaments was plotted at the starting time of its velocity measurement. Approximately 1 min after addition of nucleotide(s), the average sliding velocity of the individual filament was determined as follows: in order to prevent photodamages, the measuring field under vision was changed frequently. In 2 mM GTP, 27 filaments in 11 fields. For a given filament, the average sliding velocity was determined from the displacement in a 30 s time span followed by a 10 s time interval. In 15μM ATP, 40 filaments in 9 fields. In 15 μM ATP plus 2 mM GTP, 42 filaments in 10 fields was the average in a 6 s time span followed by a 2 s time interval. The solid lines were the result of linear regression for both these data sets. The dashed lines were our preliminary results [10]. The deceleration observed in 15 μM ATP in the absence of ATP-regenerating system only was statistically significant ($P < 0.1\%$).

Condition	Velocity at $t = 0$ μm/s **	slope μm/s/min ***
minus ATP-regenerating system * (dashed line)		
(2) + 15μM ATP, 2mM GTP ($n = 42$)	0.74 ± 0.04	-0.002 ± 0.005
(4) + 15μM ATP ($n = 47$)	0.64 ± 0.03	-0.027 ± 0.004
(6) + 2mM GTP ($n = 26$)	0.08 ± 0.01	-0.0002 ± 0.0008
plus ATP-regenerating system (solid line)		
(1) + 15μM ATP, 2mM GTP ($n = 42$)	0.74 ± 0.06	-0.001 ± 0.007
(3) + 15μM ATP ($n = 40$)	0.59 ± 0.04	-0.0009 ± 0.0043
(5) + 2mM GTP ($n = 27$)	0.065 ± 0.009	-0.0001 ± 0.0005

* Ref. [10].

** The velocity at $t = 0$ was obtained by extrapolation to $t = 0$. This was represented as the velocity and its standard error.

*** The slope was calculated using linear regression of each data. This was represented as the regression coefficient and its standard error.

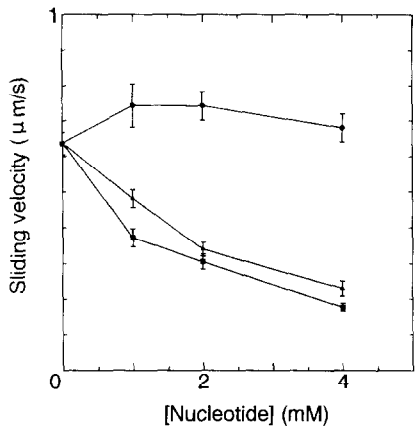

Fig. 7. Effect of varying the concentration of GTP(γS), GDP and GTP (0–4 mM) on the sliding velocity of actin filament in the presence of 15 μM ATP in the absence of ATP-regenerating system. The movement of filament was observed in SBL solutions containing 15 μM ATP plus GTP(γS) (■), GDP (▲) or GTP (●). Deceleration of sliding velocity with time in 15 μM ATP without ATP-regenerating system disappeared by adding of 2 mM GTP(γS), while the deceleration remained in the case of 2mM GDP. The sliding velocity obtained at the extrapolation to $t = 0$ after linear regression and its standard error ($n = 25$–48) was then plotted.

the surface-fixed H-meromyosin. GTP(γS) induced a dissociation of H-meromyosin from F-actin according to light scattering measurement (data not shown). The sliding velocity in 15 μM ATP was reduced with the increasing amount of coexisting GTP(γS). A similar effect was observed with GDP that could not support acto-H-meromyosin motility (Fig. 7). These results suggested that the ability (of H-meromyosin heads with GTP bound) to drive actin filament was requisite to the increase in the sliding velocity by addition of GTP.

As a result, the mutual sensitization of the driving activity between ATP and GTP admits an explanation that the displacement of actin filament by an H-meromyosin head increases by the neighboring heads that are driving it.

4. Discussion

The elementary reaction site of actomyosin motility resides in the ternary complex of actin-myosin-ATP. It is reasonable to consider that in an in vitro motility assay of actomyosin, the sliding velocity (V) of a single actin filament of a given length (L) is determined as a function of the total number of elementary reaction sites along the filament which is equal to the number of myosin heads interacting with the actin filament (m). Apparently the number (m) is a function of both the density of myosin heads along the actin filament (m/L) and the ATP concentration in the solution. Knowledge of the relationship between m and V, in other words, of the integration over the multiple elementary reactions along a single actin filament, is essential in order to understand the molecular mechanism of the sliding motility of actomyosin.

Among important findings so far obtained in in vitro motility studies of the actomyosin system, we wish to take the following into consideration; (1) Under the condition where the active sites of myosin heads are saturated with ATP, the sliding velocity (V) of actin filament increased with the density of myosin heads along the filament (m/L) [7,27], and finally V became constant at the high density [7,27]. That is, V depended on m/L. (2) Under the condition of saturation of ATP binding to myosin active sites at any density of myosin heads, the sliding velocity of actin filament (V) is essentially independent of the length of filament (L) [7–9,27]. In other words, V is not directly related to the total number (m) interacting with the filament.

These two facts are summarized as follows; The sliding velocity of an actin filament is primarily determined as a function of the average distance $\langle d \rangle$ between active myosin heads along actin filament in such a way that V increases with decreasing $\langle d \rangle$. Apparently $\langle d \rangle$ is just the inverse of the myosin head density along the actin filament, $\langle d \rangle = \langle L/m \rangle$. Furthermore, a critical length of $\langle d \rangle$ is likely to be present where V becomes constant even with further increase in myosin density, which we denote as $\langle d \rangle_{cr}$. In a standard experiment made by Uyeda at al. [7] of in vitro motility assay of actomyoisn at the saturation of ATP, the maximum velocity was essentially maintained down to an average density of approximately 27 H-meromyosin (approximately 50 heads) per 1 μm of actin filament. This may indicate that $\langle d \rangle_{cr}$ is of the order 20–40 nm.

When the average distance between the active heads $\langle d \rangle$ is long at the low concentration of ATP,

the increase in ATP concentration decreases the average distance. From this viewpoint, the mutual sensitization between ATP and GTP may admit an interpretation that GTP also contributes in decreasing the average distance in cooperation with ATP. On the whole, the critical average distance intrinsic to GTP may not be the same as that of ATP. This finding may add an important aspect with regard to the integration over the neighboring elementary reactions.

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