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# Effect of deuterium oxide on actomyosin motility in vitro

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**Abstract**

Actin filament velocities in an in vitro motility assay system were measured both in heavy water (deuterium oxide, $D_2O$) and water ($H_2O$) to examine the effect of $D_2O$ on the actomyosin interaction. The dependence of the sliding velocity on pD of the $D_2O$ assay solution showed a broad pD optimum of around pD 8.5 which resembled the broad pH optimum (pH 8.5) of the $H_2O$ assay solution, but the maximum velocity ($4.1 \pm 0.5$ μm/s, $n = 11$) at pD 8.5 in $D_2O$ was about 60% of that ($7.1 \pm 1.1$ μm/s, $n = 11$) at pH 8.5 in $H_2O$. The $K_m$ values of 95 and 80 μM and $V_{max}$ values of 3.2 and 5.1 μm/s for the $D_2O$ and $H_2O$ assay were obtained by fitting the ATP concentration dependence of the velocity (at pD and pH 7.5) to the Michaelis–Menten equation. The $K_m$ value of actin-activated Mg-ATPase activity of myosin subfragment 1 (S1) was decreased from 50 μM [actin] in $H_2O$ to 33 μM [actin] in $D_2O$ without any significant changes in $V_{max}$ (9.4 $s^{-1}$ in $D_2O$ and 9.3 $s^{-1}$ in $H_2O$). The rate constants of ADP release from the acto-S1–ADP complex measured by the stopped flow method were $361 \pm 26$ $s^{-1}$ ($n = 27$) in $D_2O$ and $512 \pm 39$ $s^{-1}$ ($n = 27$) in $H_2O$ at 6°C. These results suggest that the decrease in the in vitro actin-myosin sliding velocity in $D_2O$ results from a slowing of the release of ADP from the actomyosin–ADP complex and the increase in the affinity of actin for myosin in the presence of ATP in $D_2O$. 

*Keywords:* Deuterium oxide; Actomyosin; In vitro motility assay

## 1. Introduction

Sliding movement between actin and myosin filaments in muscle contraction is generally thought to occur as the result of a cyclic association and dissociation of myosin heads with actin-binding sites involving ATP hydrolysis (for reviews see [1–3]). In studying the interaction between actin and myosin, an in vitro motility assay system which visualizes the movement of fluorescently labeled actin filaments over a myosin-coated glass surface has been shown to be useful, and in vitro actin filament sliding has been studied extensively under various conditions, ionic strength, Mg-ATP concentration, temperature and pH, to examine the determinant of the velocity [4–10].

In the present work, we have examined the effect of substituting $D_2O$ for $H_2O$ on the in vitro actin filament sliding velocities. Water is known to be involved in the myosin ATP hydrolysis step ($MATP \rightarrow MADPP_i$), in which the water attacks the γ-phosphorus atom of ATP [11], and besides the active role as the reactant of ATP hydrolysis, the hydration of motor proteins in determining the kinetics has also been studied [12,13]. Hotta and Morales [14] and Inoue et al. [15] reported that myosin ATPase activity in $D_2O$ decreased to about 60% of

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that in $H_2O$. Experiments with skinned muscle fiber preparations have shown that at saturating calcium concentrations the muscle fiber generates about 20% more isometric force in $D_2O$ than in $H_2O$ [16]. It remains unclear, however, how $D_2O$ affects the acto-myosin interaction. We have demonstrated here that the actin filament velocity in the in vitro motility assay was about 40% slower in $D_2O$ than in $H_2O$. The $K_m$ value of the actin-activated Mg-ATPase activity of S1 was 33% smaller in $D_2O$ than in $H_2O$ without changing the $V_{max}$ values in $D_2O$ and $H_2O$, and the rate of ADP dissociation from the acto-S1–ADP complex measured by the stopped flow method was about 30% smaller in $D_2O$ than in $H_2O$. Possible causes for the slower sliding velocity in $D_2O$ are discussed. Preliminary results have been presented in abstract form [17].

## 2. Materials and methods

### 2.1. Materials

Myosin and actin were prepared from rabbit skeletal muscle by the method of Perry [18], and Spudich and Watt [19], respectively. Myosin was stored in 50% glycerol at −20°C. Heavy meromyosin (HMM) and S1 were prepared as described by Okamoto and Sekine [20]. $D_2O$ (99.9%) was from Aldrich (Milwaukee, WI, USA). ATP, bovine serum albumin, glucose oxidase and catalase were from Sigma (St. Louis, MO, USA). Other chemicals were of analytical grade.

### 2.2. In vitro motility assay

The sliding velocity of fluorescent actin filament was measured as described by Chaen et al. [21] with some modifications. After allowing the rhodamine-phalloidin-labeled actin filaments to bind to the HMM (80 μg/ml) over the nitrocellulose-coated glass coverslips in an ATP-free solution (25 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.04% $NaN_3$, and 25 mM imidazole (pH7.5)), assay solutions were introduced into the flow cell to start the movement of actin filaments. The temperature in the flow cell was adjusted by circulating temperature-controlled water into a brass block attached to the microscope stage and another block jacketing the objective. Experiments were carried out at 25°C. The assay solutions contained 25 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.04% $NaN_3$, 4.5 mg/ml glucose, 0.22 mg/ml glucose oxidase, 0.036 mg/ml catalase, 2 mM ATP, and 25 mM imidazole (pH 7.5 and pD 7.5 for the $H_2O$ and $D_2O$ assay solution, respectively). The $D_2O$ assay solution was prepared by dissolving the salts in 99.9% $D_2O$, and the pD was adjusted by adding a $D_2O$ solution of DCl or KOH until a pD with the relationship pD = pH reading+0.4 was reached [22].

### 2.3. ATPase measurements

Actin-activated Mg-ATPase activity of S1 was measured in the $H_2O$ and $D_2O$ assay solutions using 0.25 μM S1 at various concentrations of actin (2.5–30 μM) at 25°C. $D_2O$ content in the solution was about 98%; the protein sample in $D_2O$ was prepared using S1 (12.5 μM) in $H_2O$, and F-actin (140 μM) in $D_2O$ prepared by homogenizing the pelleted F-actin. The protein sample was incubated on ice for more than 12 h. Liberated inorganic phosphate was determined by the malachite green method [23].

### 2.4. Kinetic measurements

Measurement of ADP release from acto-S1 followed the procedure of White [24], using a stopped flow spectrometer (Applied Photophysics SX18MV, Leatherhead, UK). Since the acto-S1 dissociation by ATP in the presence of ADP is rate-limited by ADP release, the rate of ADP release was determined from the acto-S1 dissociation, which is detected in the decrease in light scattering (340 nm light scattered at 90° from the incident beam) when acto-S1–ADP (2 μM actin, 1.5 μM S1, and 50 μM ADP) was mixed with 2 mM ATP. Preparation of the protein sample in $D_2O$ was similar to the above except for 25 μM S1, and 97% $D_2O$ content. We performed the measurements at low temperature, 6°C, since preliminary experiments at 20°C showed a rate of $\sim$1000 $s^{-1}$, which is near the limit of the stopped flow measurement, and the process has been reported to be temperature-dependent and the Arrhenius plot was linear from 0°C to 25°C [25].

## 3. Results

### 3.1. Sliding velocities of actin filaments on HMM

The rate of an enzymatic reaction generally depends on hydrogen ion concentration. First we examined whether there were differences between the pH and pD dependence of the actin filament velocity. Fig. 1 shows the dependence of the actin sliding velocity on pH and pD of the $H_2O$ and $D_2O$ assay solution, respectively. In the experiment, different pH(pD) buffer reagents were used: 25 mM imidazole at pH 6.0, 6.5, 7.0, 7.5 and pD 6.0, 6.5, 7.0, 7.5; 25 mM Tris at pH 8.0, 8.5 and pD 8.0, 8.5; 25 mM glycine at pH 9.0, 9.5 and pD 9.0, 9.5. The sliding movement showed a broad pD optimum of around pD 8.5 which resembled the broad pH optimum (pH 8.5) of the $H_2O$ assay solution, but the maximum velocity ($4.1 \pm 0.5$ μm/s, $n = 11$) at pD 8.5 in $D_2O$ was about 60% of that ($7.1 \pm 1.1$ μm/s, $n = 11$) at pH 8.5 in $H_2O$. These results indicate that the effect of deuterium ion concentration on the actin filament movement is similar to the case of hydrogen ions except for the slower velocities in $D_2O$. The dependence is somewhat different from prior studies on the in vitro motility assay [4,8] in which the dependence has a shoulder between pH 7.5 and 8.0. Although the optima were around pH and pD 8.5, the experiments described below were carried out at pH and pD 7.5, which have been used in most of the in vitro motility assay studies.

The sliding velocity of actin filament in the in vitro motility assay has been shown to be dependent on the concentration of Mg-ATP [4,6,7,9]. Fig. 2 shows the effect of varying the Mg-ATP concentration from 20 μM to 2 mM on the actin filament velocity in $H_2O$ and $D_2O$. The data were fitted with the Michaelis–Menten equation, yielding $K_m$ values of 95 and 80 μM and $V_{max}$ values of 3.2 and 5.1 μm/s for the $D_2O$ and $H_2O$ assay, respectively. These results demonstrate that $D_2O$ just scales down the Mg-ATP concentration dependence of the actin filament velocity in $H_2O$.

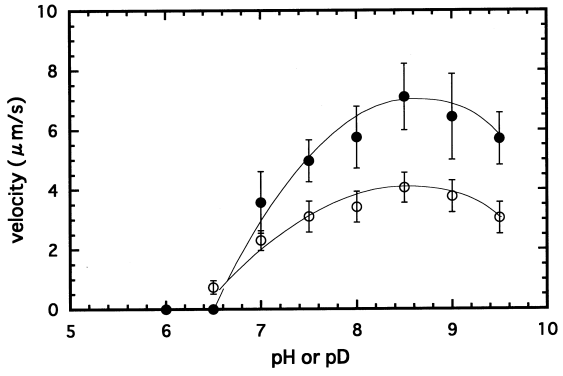


Fig. 1. Dependence of the actin sliding velocity on pH (●) and pD (○) of the $H_2O$ and $D_2O$ assay solution, respectively. Each data point represents the average velocity of 11 different filaments, and the vertical bar shows the standard deviation. The curves were drawn by eye.

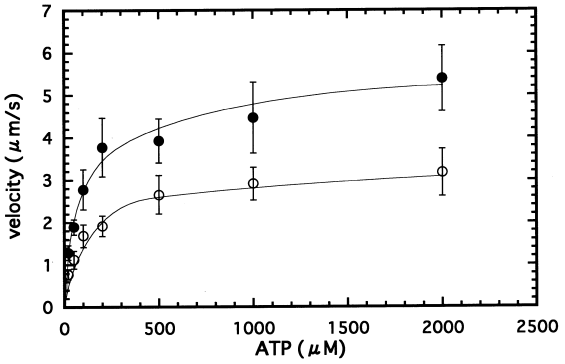


Fig. 2. Dependence of the actin sliding velocity on Mg-ATP concentration in $H_2O$ (●) and $D_2O$ (○). Each data point represents the average velocity of 11 different filaments, and the vertical bar shows the standard deviation. Curves are fit to the Michaelis–Menten equation, which gave $K_m$ values of 80 and 95 μM and $V_{max}$ of 5.1 and 3.2 μm/s for the $H_2O$ and $D_2O$ assay, respectively.

### 3.2. Actin-activated Mg-ATPase activity of S1

To examine whether $D_2O$ affects the interaction between actin and S1 in the presence of ATP, the actin-activated Mg-ATPase activity of S1 was studied. The Mg-ATPase activities of S1 as a function of actin concentration both in $D_2O$ and $H_2O$ are shown in Fig. 3. Fits to the Michaelis–Menten equation show that the $V_{max}$ value in $H_2O$ (9.3 $s^{-1}$) was scarcely altered in $D_2O$ (9.4 $s^{-1}$), while the $K_m$ value was decreased from 50 μM in $H_2O$ to 33 μM in $D_2O$. These data indicate that $D_2O$ affects the affinity of actin for S1 in the presence of ATP without changes in the maximum rate of the reaction.

### 3.3. Rate of ADP release from acto-S1

ADP release from the cross-bridge has been be-

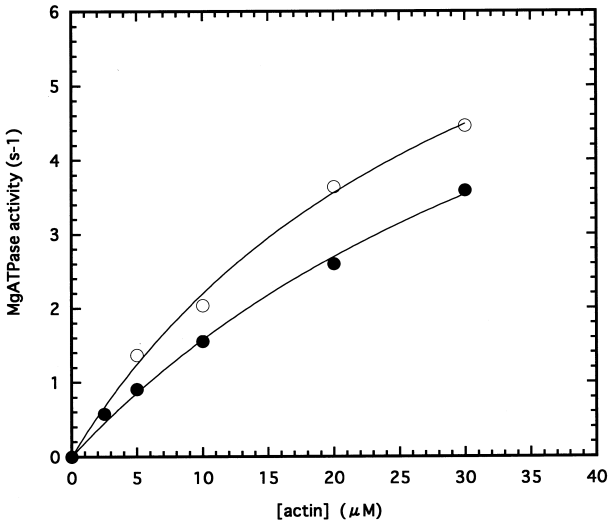


Fig. 3. Mg-ATPase activity as a function of actin concentration in $H_2O$ (●) and $D_2O$ (○). Curves are fit to the Michaelis–Menten equation, which gave $K_m$ values (actin concentration at which the Mg-ATPase activity is half of its maximum) of 50 and 33 μM and $V_{max}$ of 9.3 $s^{-1}$ and 9.4 $s^{-1}$ in the $H_2O$ and $D_2O$ assay solution, respectively.

lieved to the limit unloaded shortening velocity [25]. Fig. 4 shows acto-S1 dissociation in the presence of ADP, the rate of which is the same as that of the ADP release, since the ADP release limits the acto-S1 dissociation [24]. The rate constant of ADP release was 30% slower in $D_2O$ ($361 \pm 26$ $s^{-1}$ ($n = 27$)) than in $H_2O$ ($512 \pm 39$ $s^{-1}$ ($n = 27$)) at 6°C. The difference was significant ($P < 0.0001$).

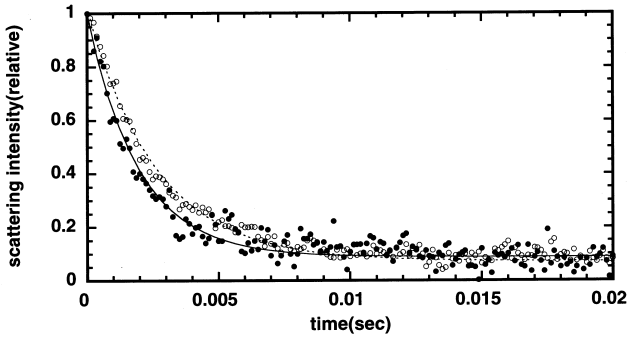


Fig. 4. Changes in light scattering intensity on mixing acto-S1–ADP (2 μM actin, 1.5 μM S1, and 50 μM ADP) with 2 mM ATP measured by a stopped flow spectrometer. Solid and open symbols are the data points in $H_2O$ and $D_2O$ assay solution, respectively. Straight and dotted curves are the best fit to a single exponential equation, which gave a rate constant of 510 $s^{-1}$ in $H_2O$ and 377 $s^{-1}$ in $D_2O$, respectively.

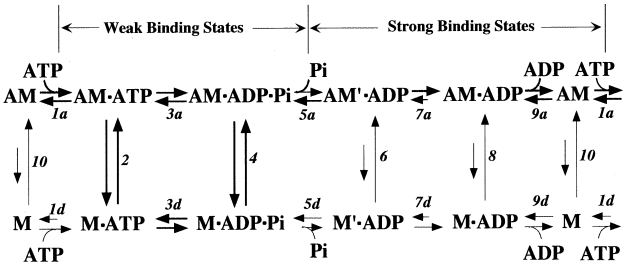


Fig. 5. Kinetic scheme of the actomyosin ATPase, where A and M represent actin and myosin head, respectively. (Modified from [3].)

## 4. Discussion

In the present experiments, we have shown that the actin filament velocity in the in vitro motility assay was about 40% slower in $D_2O$ than in $H_2O$, $D_2O$ did not affect the $V_{max}$ values, but decreased the $K_m$ value for actin-activated Mg-ATPase activity of S1 to 66% of that in $H_2O$, and the rate of ADP dissociation from acto-S1–ADP complex was about 30% smaller in $D_2O$ than in $H_2O$.

### 4.1. Effects of $D_2O$ on actomyosin kinetics

$D_2O$ is known to have a higher viscosity, melting point, heat capacity and temperature of maximum density than $H_2O$ [26]. These properties suggest that the degree of hydrogen bonding is higher in $D_2O$. Recent crystallographic data [27] have indicated that Mg-ADP and metallofluoride, a phosphate analogue, are held in the nucleotide-binding pocket of the myosin motor domain through hydrogen bonds. When the surrounding medium of the myosin head is changed from $H_2O$ to $D_2O$, an exchange between hydrogen in the protein and deuterium of $D_2O$, and also an exchange between the ordered water in the pocket and $D_2O$ are expected to occur. Yamada et al. [28] showed that indole amide hydrogens of tryptophan residues in the myosin head incubated in $D_2O$ were exchanged with deuterium at a rate of $\sim 1$ $s^{-1}$. These data suggest that the exchange between hydrogen and deuterium in the nucleotide pocket would raise the degree of hydrogen bonding to hold ADP, which results in a slower rate of ADP release from the acto-S1–ADP complex (Fig. 5, step 9a) and a higher rate of ATP cleavage in the myosin head (Fig. 5, step 3d). The lower steady-state

Mg-ATPase activity of myosin (Fig. 5, step 5d) in $D_2O$ than in $H_2O$ [14,15] is accounted for by assuming that phosphate ion is also held tighter in the pocket in $D_2O$ than in $H_2O$. The effect of $D_2O$ on the actin-activated Mg-ATPase activity of S1 is discussed in Section 4.2.

### 4.2. Effects of $D_2O$ on the actin filament sliding velocity and its kinetic determinant

The frictional drag force against the sliding actin filament is calculated to be about $10^{-14}$ N (the frictional drag force $f=\gamma v$, where $v\cong 10$ μm/s, and $\gamma$ is the frictional coefficient, $\gamma=2\pi\eta L/\ln(2h/r)$ [29] for $\eta=0.01$ g/cm/s, the length of actin filament, $L\cong 1$ μm, the height at which the filament is sliding above a surface $h\cong 10$ nm, and the radius of the filament $r=5$ nm), which is much smaller than the maximal force of one cross-bridge ($\cong 10^{-12}$ N) [11,30]. So the slower velocity of the actin filament in $D_2O$ cannot be accounted for by the 25% increase (at 25°C) in the viscosity of $D_2O$ compared with that of $H_2O$ [26] but by some rate determining step which decreased in the $D_2O$ assay solution.

In 1985, Siemankowski et al. [25] suggested that the rate of ADP release can limit the unloaded shortening velocity of muscle, in contrast to the previous suggestion of Barany [31] that the rate of ATP hydrolysis by actomyosin limits the unloaded shortening velocity. Our in vitro data in $D_2O$ are consistent with those of Siemankowski et al. that the actin filament sliding velocity does not correlate with $V_{max}$ of actin-activated Mg-ATPase activity of S1 but with the rate of ADP release from actomyosin. Namely, the decrease in actin filament velocity in $D_2O$ is due to the higher degree of hydrogen bonding to hold the ADP in the pocket, which results in the slowing of the ADP release from actomyosin (Fig. 5, step 9a).

Another cause for the slower sliding velocity in $D_2O$ may be apparent from the $K_m$ value (the parameter of the affinity of actin for myosin in the presence of ATP) of the actin-activated Mg-ATPase activity of S1. The $K_m$ value for the actin-activated Mg-ATPase activity of S1 was 33 μM in $D_2O$ and 50 μM in $H_2O$. Recently, Amitani et al. [32] have deduced that the in vitro sliding velocity is proportional to the value of $(V_{max}\ K_m)^{1/2}$ from their force-balance model, and shown that in vitro velocity data [33–39] were well fitted to this equation. In their model, the sliding velocity is determined by the balance between the force from active cross-bridges and the resistive drag force mainly from weakly bound cross-bridges (shown as weak binding states in Fig. 5). The parameter of $K_m$ is included in the equation to express the time-averaged drag force on the assumption that the $K_m$ approximately equals the dissociation constant of weakly bound cross-bridges. Our in vitro and solution kinetics data fit to their equation, implying that $D_2O$ might affect the equilibrium constant (steps 2 and 4 in Fig. 5) of weakly bound cross-bridges so that the resistive drag force is increased.

The possible causes for the slower sliding velocity in $D_2O$ are summarized as follows. (a) The decrease in the ADP release rate (step 9a in Fig. 5). (b) The increase in the affinity of F-actin for myosin at the weak binding state (steps 2 and 4 in Fig. 5). In order to reconcile the decrease in ADP release rate in $D_2O$ with the decrease in the $K_m$ value in $D_2O$, it would be natural to assume that $K_m$ in the equation includes the post-power stroke state (AMADP state in Fig. 5) in addition to the weak binding state, although the fraction of the post-power stroke state is calculated to be very small [32,40]. Sweeney et al. [41] have shown that the actin sliding velocity was altered by the rate of ADP release but was not solely determined by it in their study on a surface loop (25/50 kDa loop), suggesting that another step in addition to the ADP release step may contribute to the rate of unloaded actin-based movement. Measurement of the stiffness–speed relationship of muscle fiber, which revealed the amount of attached cross-bridges in the relaxed state [42], would elucidate whether $D_2O$ affects the equilibrium in the weak binding states in addition to the ADP release step.

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