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# Sucrose increases the activation energy barrier for actin–myosin strong binding

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ABSTRACT

To determine the mechanism by which sucrose slows in vitro actin sliding velocities, *V*, we used stopped flow kinetics and a single molecule binding assay, SiMBA. We observed that in the absence of ATP, sucrose (880 mM) slowed the rate of actin–myosin (A–M) strong binding by 71 ± 8% with a smaller inhibitory effect observed on spontaneous rigor dissociation (21 ± 3%). Similarly, in the presence of ATP, sucrose slowed strong binding associated with $P_i$ release by 85 ± 9% with a smaller inhibitory effect on ATP-induced A–M dissociation, $k_T$ (39 ± 2%). Sucrose had no noticeable effect on any other step in the ATPase reaction. In SiMBA, sucrose had a relatively small effect on the diffusion coefficient for actin fragments (25 ± 2%), and with stopped flow we showed that sucrose increased the activation energy barrier for A–M strong binding by 37 ± 3%, indicating that sucrose inhibits the rate of A–M strong binding by slowing bond formation more than diffusional searching. The inhibitory effects of sucrose on the rate of A–M rigor binding (71%) are comparable in magnitude to sucrose's effects on both *V* (79 ± 33% decrease) and maximal actin-activated ATPase, $k_{cat}$, (81 ± 16% decrease), indicating that the rate of A–M strong bond formation significantly influences both $k_{cat}$ and *V*.



## Introduction

Muscle contraction is generated through the A–M ATPase cycle (Fig. 1A), which modulates A–M affinity between weak- and strong-binding states. A–M binding occurs first through a weak-binding equilibrium, $K_{WS}$, followed by strong bond formation, $k_{SB}$ (Fig. 1A). But because the techniques used herein are only sensitive to strong bond formation, in our analysis we assume that A–M strong binding occurs as a single step having an effective rate constant $k_{att(+ATP)} = K_{WS} \cdot k_{SB}$ (Fig. 1A). During muscle contraction, A–M strong bond formation is associated with a myosin lever arm rotation and phosphate, $P_i$, release [1–3]. This mechanochemical step is the molecular mechanism for force generation in muscle [1,4–6] and is thought to be rate-limiting for actin-activated ATPase activity [7]. A–M strong binding can also occur in the absence of nucleotide, and again here we assume in our analysis a single rigor-binding step having an effective rate constant $k_{att(-ATP)}$ (Fig. 1B). A–M detachment occurs upon ATP binding to myosin with a second-order rate constant $k_T$ (Fig. 1A). In the absence of nucleotide, A–M detachment can occur spontaneously with a rate $k_{det(-ATP)}$ (Fig. 1B).

Known inhibitors of A–M strong binding such as BTS (N-benzyl-p-toluene sulphonamide), BDM (2,3-butanedione monoxime), and blebbistatin decrease $k_{SB}$ by slowing $P_i$ release [8–10]. Specifically, they affect $k_{att(+ATP)}$ (Fig. 1A) but not $k_{att(-ATP)}$ (Fig. 1B). In order to determine the effects of strong A–M binding on A–M ATPase biochemistry and mechanics, an inhibitor that specifically slows the rate of A–M strong binding, $k_{att(-ATP)}$, is needed. Here we show that sucrose is such an inhibitor.

Sucrose inhibits the force generated by skinned muscle fibers [11] and slows in vitro actin sliding velocities, *V* [12]. Sucrose has also been used to probe the kinetics of non-muscle myosins [13]. For myosin V and VI, De La Cruz and coworkers showed that sucrose slows ADP binding and detachment without affecting the ADP dissociation constant, $K_{ADP}$ (Fig. 1A). Although it has no known physiological significance, sucrose is an accessible, stable, and reversible [14] reagent that is useful for studying the relationship between A–M kinetics and mechanics. To date, the mechanism by which sucrose inhibits muscle contraction and *V* remains unclear.

Two possible mechanisms for inhibition of muscle mechanics by sucrose are mechanical (viscous) and chemical (ATPase). It has been argued that sucrose does not inhibit *V* by imposing a mechanical load on the actin filament [8], and data presented herein support this argument (Fig. 2B and Fig. 3). It has also been shown that

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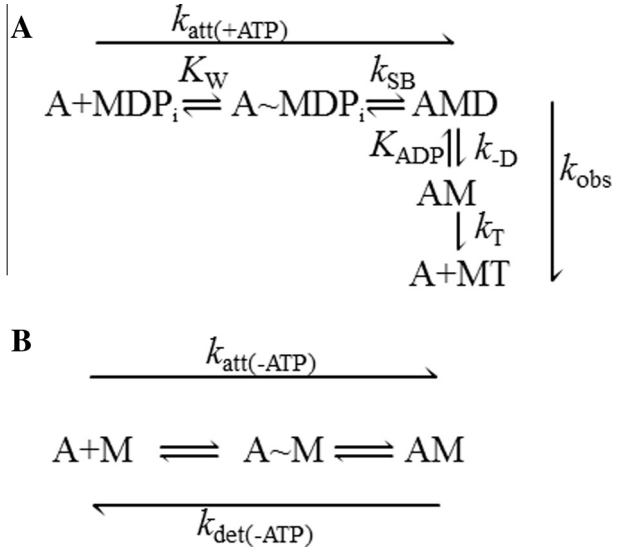


**Fig. 1.** Kinetic schemes of strong A–M binding in the (A) presence and (B) absence of ATP. (A) In the presence of MgATP, A–M strong binding is a two-step process. Weak actin–myosin binding (A ~ MDP$_i$) is thought to occur rapidly with an equilibrium binding constant $K_W$. Strong A–M binding, with a rate constant $k_{SB}$, is associated with $P_i$ release and a myosin lever arm rotation. The effective rate constant for this two-step binding reaction is $k_{att(+ATP)} = K_W \cdot k_{SB}$. ADP release from A–M occurs with a rate constant $k_{-D}$ followed by ATP-induced A–M detachment with a second-order rate constant $k_T$. (B) Even in the absence of ATP, A–M strong binding occurs through a two-step reaction with an effective rate constant, $k_{att(-ATP)}$. A–M detachment can occur spontaneously with a rate constant $k_{det(-ATP)}$. A = actin, D = MgADP, T = MgATP, $P_i$ = phosphate, M = myosin.

sucrose has no significant effect on myosin (basal) ATPase activity [9], implying that sucrose does not slow product release in the absence of actin. The effect of sucrose on ATPase activity in the presence of actin has not been previously tested. It has been suggested that sucrose inhibits ADP release from the A–M complex [12]. Here we show that sucrose slows $k_{att(-ATP)}$ and to a lesser extent the rate of A–M dissociation without significantly affecting the ADP release rate.

In this paper, using both single molecule and bulk kinetic assays, we show that 880 mM sucrose inhibits A–M strong binding, slowing both $k_{att(+ATP)}$ (Fig. 1A) and $k_{att(-ATP)}$ (Fig. 1B) by 70–85%. Sucrose had a relatively small effect on the diffusion coefficient for actin fragments in our single molecule binding assay, SiMBA, and increased the activation energy barrier for A–M strong binding, indicating that sucrose inhibits the rate of A–M strong binding by slowing bond formation more than diffusional searching. The 85% inhibition of A–M binding resembles measured effects of sucrose both on the maximal actin-activated ATPase activity $k_{cat}$ (81%) and on $V$ (79%), indicating that the rate of A–M strong bond formation significantly influences both $k_{cat}$ and $V$.

## Materials and methods

### *Protein purification*

Skeletal muscle myosin was prepared from rabbit psoas muscle as previously described and stored in 50% glycerol at −20 °C [15,16]. Subfragment-1 (S1) was prepared by either chymotryptic or papain digestion of myosin [16,17]. A myosin buffer of 300 mM KCl, 25 mM imidazole, 1 mM EGTA, and 4 mM $MgCl_2$ was used to dilute myosin and S1 to experimental concentrations for use in in vitro motility and single molecule binding assays. For transient kinetic experiments, S1 was diluted in 23 mM imidazole (pH 7.4), 85 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM

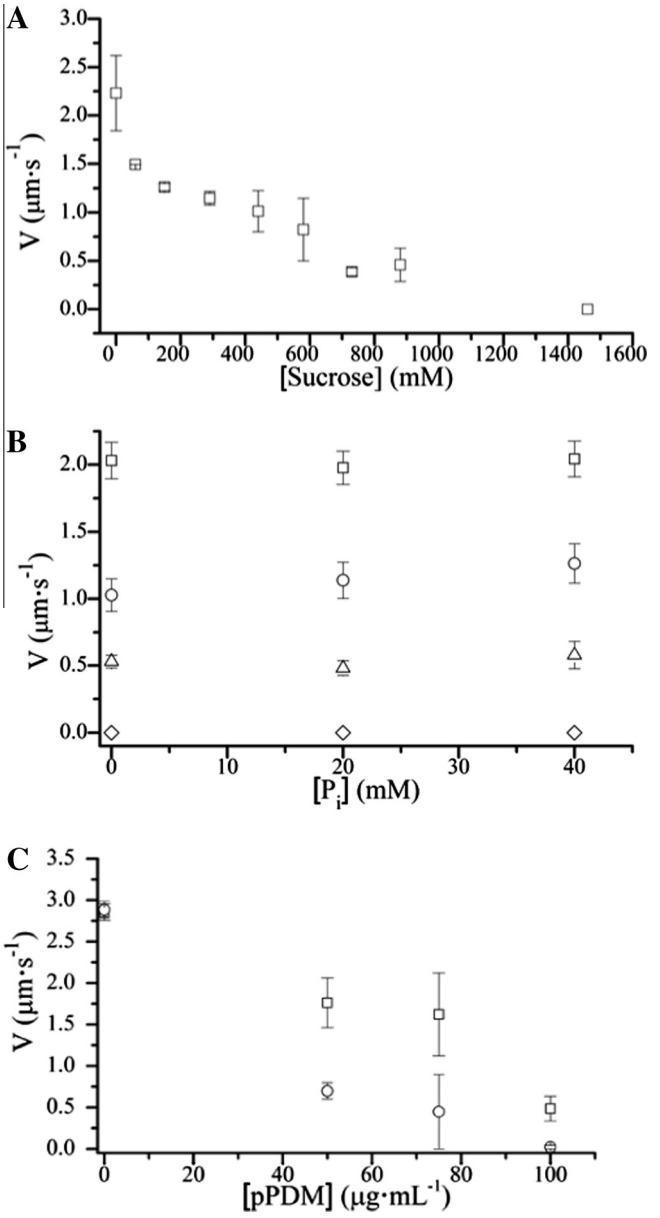


**Fig. 2.** The effects of sucrose and phosphate, $P_i$, on actin sliding velocities, $V$. (A) 880 mM sucrose decreased $V$ by 80%. (B) The addition of 20 or 40 mM $P_i$ had no effect on actin sliding velocities at 0 (□), 290 (○), 730 (△), and 1460 mM (◇) sucrose, indicating that sucrose does not slow $V$ through a mechanical load. (C) pPDM-modified myosin adsorbed to the motility surface imposes a mechanical load that slows $V$. Increasing pPDM slowed $V$ more in the presence of 30 mM $P_i$ (○) than in the absence of $P_i$ (□), showing that $P_i$ slows motility in the presence of a mechanical load.

EGTA. Actin was isolated from rabbit psoas muscle and stored on ice at 4 °C [18]. An actin buffer of 50 mM KCl, 50 mM imidazole, 2 mM EGTA, 8 mM $MgCl_2$, 10 mM DTT, and an oxygen scavenger system (292 mg mL$^{-1}$ glucose, 1.63 mg mL$^{-1}$ glucose oxidase, and 2.25 mg mL$^{-1}$ catalase) was used to dilute actin used in motility and single molecule assays.

### *Motility assay*

For in vitro motility assays, actin was incubated with a 1:1 molar ratio of TRITC (tetramethylrhodamine) phalloidin (Sigma–Aldrich, St. Louis, MO, USA) overnight at 4 °C. In vitro motility experiments with whole myosin were performed as previously described, except here we ignored in our analysis actin trajectories