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Review Article (Invited)

Unraveling the fastest myosin: Discovery history and structure-function relationships of algae *Chara* myosin XI

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Plant myosins have higher velocities than animal myosins. Among them, myosins in freshwater algae of the genus *Chara* have extremely high velocities. We have biochemically studied myosins that perform high-speed movements in the alga *Chara*. Our studies have elucidated the structural and enzymatic basis for the fast movement of *Chara* myosins. This review outlines the history leading to the discovery of the fastest myosin, algae *Chara* myosin XI, and the structure-function correlation of the fastest myosin. This review article is an extended version of the Japanese article, "Structure-function Relationship of the Fastest Myosin" by Ito et al., published in SEIBUTSU BUTSURI Vol. 63, p.91-96 (2023).

Key words: motor protein, cytoplasmic streaming, actin, crystal structure analysis

Significance

It has been predicted that the fastest myosin in the biological world exists in alga *Chara*, but its identity has remained unknown. Recently, we succeeded in cloning the fastest myosin and characterized its amino acid sequence. We also succeeded in solving the first atomic structure (2.8 Å resolution) of myosin XI, the fastest myosin class, using X-ray crystallography. Based on this crystal structure and mutation experiments, it appears that the actin-binding region contributes to the fast movement of *Chara* myosin.

Introduction

We have been studying the myosins of *Characeae* algae known for their exceptionally fast cytoplasmic streaming. Recently, we cloned four myosin XIs (*Cb*XI-1, 2, 3, 4) predicted from genome projects on *Chara braunii*, expressed them in insect cells, and purified them. Our findings revealed that all four myosin XIs exhibit higher velocities than any other known myosin from both animals and plants. Notably, *Cb*XI-1 was identified as the fastest myosin in the biological world [1]. *Cb*XI-4 is an ortholog of the previously identified fast myosin, *Chara corallina* myosin XI (*Cc*XI). Using *Cc*XI, we elucidated the regions and amino acid sequences responsible for its fast movement [2] and detailed the enzymatic kinetics enabling such fast movements [3,4]. In this review, we outline the history of myosin research and provide a comprehensive overview of our studies on fast *Chara* myosin XIs.

Myosin is a motor protein that uses the energy derived from ATP hydrolysis to move along actin filaments. In animals, myosin plays a crucial role in a wide range of movements ranging from organism-level activities such as running, flying, and jumping, to organ-level functions like heartbeats and vascular contractions. Moreover, myosin is integral to cellular-level processes including cell motility and cytoplasmic division, as well as intracellular material transport. The discovery of myosin dates back to the mid-19th century and is attributed to Wilhelm Kühne and others [5]. Subsequently, Engelhardt demonstrated that myosin possesses ATPase activity [6]. Later, Szent-Györgyi and others revealed that myosin forms a

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complex with actin and acts as a contraction protein powered by ATP [7]. For nearly five decades following its discovery, research on myosin predominantly focused on muscle myosin. However, genomic analyses conducted from the 1990s to 2000 revealed the existence of 40 myosin genes in humans [8]. Among these, 14 belong to the muscle myosin class known as "Myosin II," including skeletal, cardiac, and smooth muscle myosins, while the remaining 25 genes were identified as belonging to different myosin classes. Classification of myosin is based on the homology in the amino acid sequence of the motor domain, with the exception of myosin II, classes are assigned according to the order of their discovery. Recent genomic projects conducted in various organisms apart from humans have shown that myosin forms a superfamily of more than 79 classes, each of which contains numerous subclasses [9]. These classes and subclasses of myosin differ significantly in characteristics such as movement speed, direction, and force generated [10].

Unlike animals, which move actively in search of food, powered by muscle myosin, plants can produce energy through photosynthesis and thus do not need to move, spending their entire lives in the place where they germinate. This contrast has fostered the general perception of animals as dynamic and plants as static. However, microscopic observation of plant cells reveals a phenomenon not seen in animal cells – vigorous movement known as cytoplasmic streaming [11]. This process is driven by plant-specific myosin, myosin XI [11,12]. Myosin XI propels itself along actin filaments toward the plus end of actin, while binding to the endoplasmic reticulum via its globular tail domain (GTD) (Figure 1B, C). In plant cells, actin filaments are aligned with uniform polarity just beneath the cell membrane, facilitating unidirectional cytoplasmic streaming. The primary physiological function of myosin XI in plants is to facilitate this cytoplasmic streaming [13-19]. The velocity of cytoplasmic streaming varies among plant species, a variation that is attributed to the different velocities of myosin XI in different species.

Purification of Plant Myosin XI

This chapter outlines the history of the discovery of plant-specific myosin XI. Cytoplasmic streaming is a dynamic cellular movement that has been easily observable even with rudimentary microscopes. The phenomenon was first documented in 1774 by Corti, who observed it in the freshwater alga *Chara* (Figure 1A), which exhibits the fastest known cytoplasmic streaming in the plant kingdom, reaching speeds of up to 70 μm/s at 25°C [20]. In the 1950s, Kamiya and Kuroda noted that the speed of cytoplasmic streaming in *Chara* cells was highest near the cell membrane and decreased with distance from the membrane, suggesting that a motor driving cytoplasmic streaming is near the plasma membrane [21]. In 1974, it was discovered that actin filaments in *Chara* cells were oriented with aligned polarity just below the plasma membrane [22]. These findings suggested the existence of the ultra-fast myosin in *Chara* at 70 μm/s, which is 10 times faster than myosin in animal skeletal muscle. At that time, the existence of myosin in plants was not yet known, making this discovery particularly surprising.

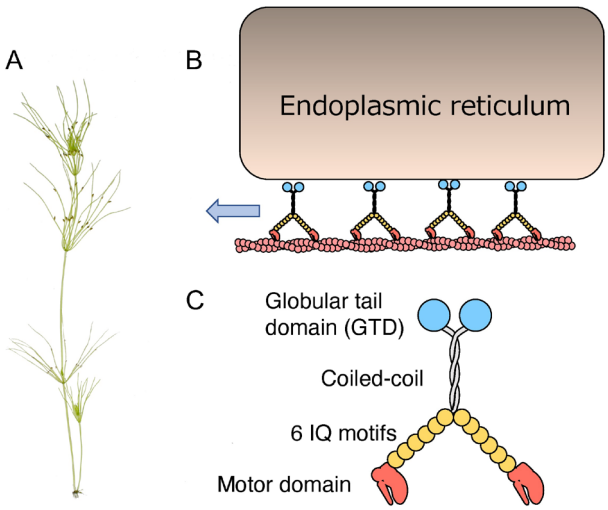

Figure 1 (A) algae *Chara* (B) Myosin XI binds to the endoplasmic reticulum at the tail and moves on actin filaments, causing cytoplasmic streaming in plant cells. (C) Schematic diagram of the domain structure of myosin XI. From the N-terminal end, a motor domain, six IQ motifs that function as lever arm, and a coiled-coil that forms a dimer, followed by a globular tail domain (GTD) that binds to the endoplasmic reticulum. This domain structure is like that of animal myosin XI. Reproduced with some modifications from Ref. [1].

However, purifying myosin from plant cells, where vacuoles rich in proteolytic enzymes occupy more than 95% of the cell volume, proved extremely challenging. It took 20 years before a successful report was published. In 1994, Yamamoto et al. succeeded in biochemically isolating myosin from *Chara corallina* using a combination of various column chromatography and *in vitro* motility assays [23]. However, the purified myosin moved at only 25 μm/s, a third of the predicted 70 μm/s based on *Chara*'s streaming speed. At the same time, Yokota et al. successfully isolated myosin from lily pollen tubes, which have fewer vacuoles [24]. The 1994 papers by Yamamoto and Yokota were the first reports of plant myosin. Due to the difficulties and low yields in plant myosin isolation, direct myosin isolation from plants has rarely been attempted since then. Cloning of the plant myosin XI gene was also delayed compared to animal myosins, with the first cloning of myosin XI from *Arabidopsis thaliana* occurring in 1993 [25]. The cloning of the fastest myosins in the biological world, moving at 70 μm/s, which was expected based on the velocity of cytoplasmic streaming in *Chara*, was not achieved until 2022 as mentioned below.

Discovery of Algae *Chara* Myosin XI

In 2000, *Chara* myosin XI (*Cc*XI) gene was cloned by immunoscreening using antibodies raised against myosin purified from *Chara corallina* [26]. We expressed and purified *Cc*XI using an insect cell expression system. The velocity of purified *Cc*XI was measured to be 22 µm/s, about one third of the 70 µm/s cytoplasmic velocity observed in *Chara,* suggesting that this *Cc*XI is not responsible for the 70 µm/s cytoplasmic streaming in *Chara* cells. However, this 22 µm/s is still three times faster than that of skeletal muscle myosin, making it the fastest myosin known at that time [4]. Using the obtained *Cc*XI, we elucidated several key aspects of its high-speed movement. We discovered that the high speed of *Cc*XI is primarily due to the significantly high ADP dissociation rate from the actin-bound myosin motor domain [3]. In addition, we found that the high velocity of *Cc*XI is dictated by its actin-binding sequence [2]. Furthermore, we also found that expression of *Cc*XI in *Arabidopsis* increased the velocity of cytoplasmic streaming and the plant size, suggesting that cytoplasmic streaming is a key determinant of plant size [16].

With the advent and widespread use of next-generation sequencing in recent years, genome projects for a wide variety of organisms have become feasible. In 2018, Nishiyama and Sakayama sequenced the genome of *Chara braunii,* a close relative of *Chara corallina*, that is also capable of the fastest cytoplasmic streaming at 70 µm/s [27]. Genome analysis suggested the presence of four myosin XI genes in *Chara braunii.* We cloned the cDNAs of these four myosin XI genes and named them *Cb*XI-1, 2, 3, and 4. A phylogenetic tree constructed from the amino acid sequences of these four motor domains revealed two subclasses. The previously reported *Cc*XI, with a velocity of 22 µm/s , was found to be an ortholog of *Cb*XI-4 in subclass 2 (Figure2). By expressing and purifying these four myosin XIs in insect culture cells and measuring their velocities using *in vitro* motility assays, we discovered that the newly cloned subclass 1's *Cb*XI-1 and *Cb*XI-2 were the long-anticipated ultra-fast myosins with velocities of 70 µm/s, with *Cb*XI-1 emerging as the fastest myosin in the biological world at 73 µm/s (Figure 3) [1]. Seventy years after Kamiya and Kuroda's 1950s prediction of the existence of the fastest myosin based on the cytoplasmic streaming in *Chara* cells, this myosin has finally been cloned, revealing its true nature.

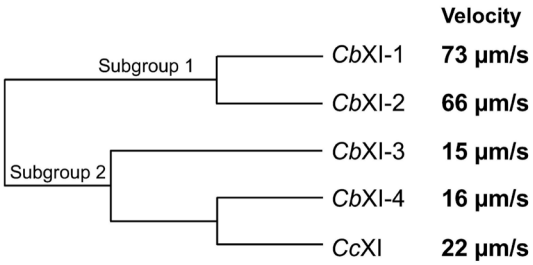

Figure 2 Phylogenetic relationships based on the amino acid sequence of the motor domain of *Chara* myosins and velocity of each Myosin. Reproduced with some modifications from Ref [1].

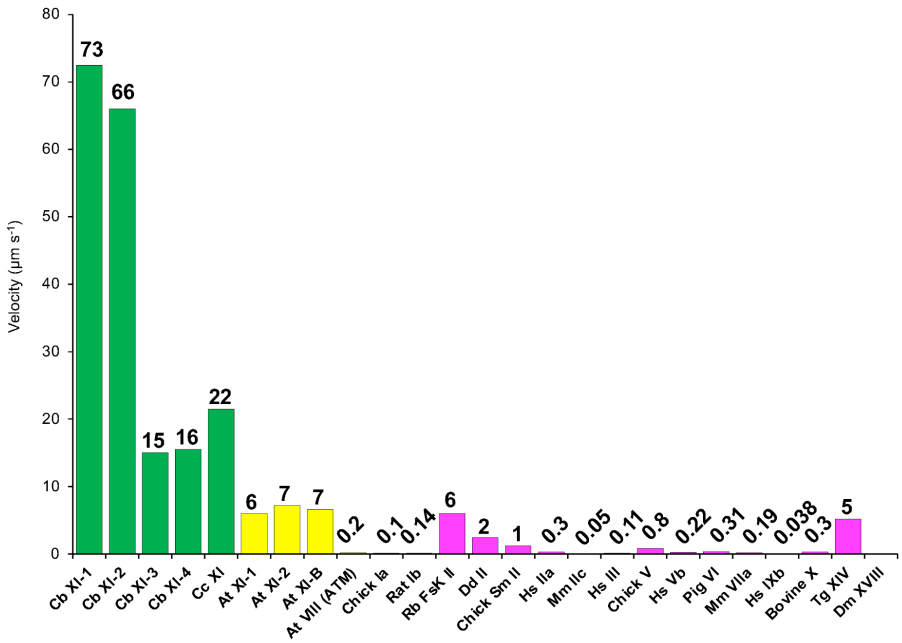

Figure 3 Velocities various classes of myosins. Green: *Chara* myosins, yellow: *Arabidopsis* myosins, magenta: animal myosins. Reproduced with some modifications from Ref. [1].

Structure-Function Relationships of Algae *Chara* Myosin XI

To understand the structural features that enable the fastest movement, we attempted to crystallize the motor domain (MD) of *Cb*XI-1 (*Cb*XI-1 MD). However, crystallization was unsuccessful, probably due to the tendency of *Cb*XI-1 to denature. Therefore, we decided to crystallize the MD of *Arabidopsis thaliana* myosin XI-2 (*At* XI-2 MD), which shares high sequence similarity (87% similarity, 63% identity) with *Cb*XI-1 MD, and construct a three-dimensional structural model of *Cb*XI-1 MD using a homology model based on the structure of *At* XI-2 MD.

At XI-2 MD, expressed and purified in insect culture cells, was incubated with ADP and AlF_4^-, which is used as an analog of ADP·Pi to mimic the pre-power stroke state of myosin, and crystallized using the sitting drop vapor diffusion method. High-resolution (2.8Å) structural data in the ADP·Pi state (ADP- AlF_4^- bound state) were successfully obtained through X-ray diffraction experiments at a wavelength of 0.98 Å. This structure represents the first high-resolution crystal structure of myosin XI, the fastest myosin class. By comparison with the structures of three different classes of animal myosin in the same ADP·Pi state, we found that although *At* XI-2 moves significantly faster, its nucleotide-binding domain structure is almost identical to that of the animal myosins compared. This suggests that the rate-limiting step for the velocity of myosin movement, ADP dissociation, is determined by structures outside the nucleotide-binding domain. Indeed, a comparison of amino acids across myosin classes revealed a high degree of conservation in the central areas of the molecule, such as the nucleotide-binding domain, while the peripheral area of the molecule showed high variability. In particular, the five actin-binding sites - loop2, loop3, loop4, CM-loop, and helix-turn-helix - exhibited extremely high amino acid variability (Figure 4).

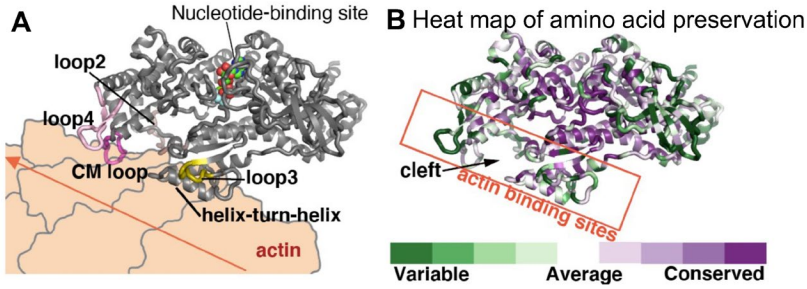

Figure 4 Actin-binding region with high amino acid diversity among myosins.(A) Docking model of *At*XI-2 MD and actin. (B) Heat map visualization of *At*XI-2 MD showing amino acid conservation and diversity. Reproduced with some modifications from Ref. [1].

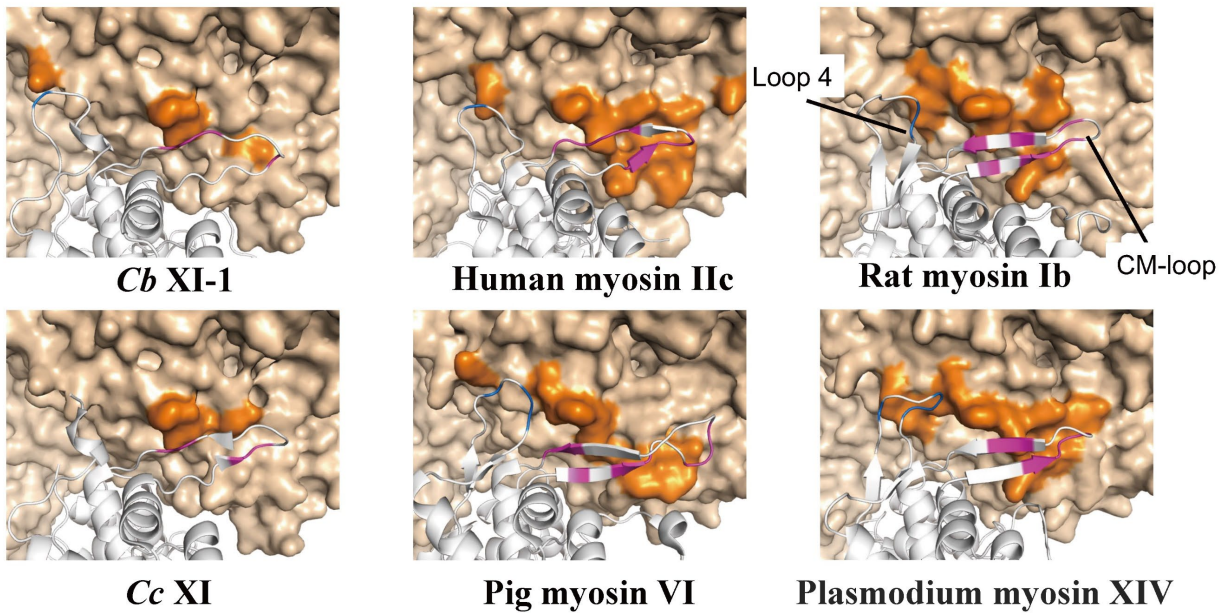

Figure 5 Interaction of CM loop and loop4 of various myosins with actin. The interactions of MDs (white) of *Cb*XI-1 (A, homology model built using PDB ID: 7KCH), *Cc*XI (B, PDB ID: 7KCH), NM2c (C, PDB ID: 5JLH), Myo6 (D, PDB ID: 6BNP), Myo1b (E, PDB ID: 6C1H), Pf MyoA (F, PDB ID: 7ALN) and actin (light orange) are shown as ribbon and surface representations. The residues in the actin, CM loop, and loop 4 within 4 Å between the actin and myosin are colored in orange, magenta, and blue. The surface model represents actin, and the ribbon model represents myosin. Reproduced with some modifications from Ref. [1].

Therefore, to investigate whether *Cb*XI-1 differs from other myosins in its binding mode to actin, we generated a structural model of *Cb*XI-1 using the homology model based on *At* XI-2 and docked it with actin. For docking, we used the recently reported cryo-electron microscopy structure of *Cc*XI - F-actin complex (PDB ID: 7KCH) [28]. Comparison of the actin-binding modes of different myosins, including *Cb*XI-1, revealed that the actin-binding modes differ significantly among different myosin classes and subclasses (Figure 5) [1].

Characteristic Sequences in Loop 2 and Loop 3 Contribute to the High-Speed Movement of *Chara* Myosin XIs

All myosin XIs of *Characeae* algae (*Chara* myosin XIs) are faster than myosins from other species (Figure 3). It was found that *Chara* myosin XIs have unique sequences in their actin-binding loops, Loop 2 and Loop 3, compared to myosins from other species. These characteristic sequences in Loop 2 and Loop 3 are key factors contributing to the high-speed movement of *Chara* myosin XIs, as discussed in the following sections.

Myosin velocity can be approximated by multiplying the step size by the inverse of the time for which myosin remains strongly bound to actin. The step size does not vary much among myosins, and it is the time myosin remains strongly bound to actin that determines myosin velocity. The time myosin remains strongly bound to actin is determined by the ADP dissociation rate from acto-myosin [29,30]. Therefore, there is a strong correlation between myosin velocities (Figure 6A) and the ADP dissociation rates from acto-myosin (Figure 6B). In contrast, the rate of ADP dissociation from myosins not bound to actin is significantly slower and exhibits minimal variation between fast and slow myosins (Figure 6C). This indicates that ADP dissociation is accelerated upon binding to actin and that this acceleration is more pronounced in faster myosins (Figure 6D) [3].

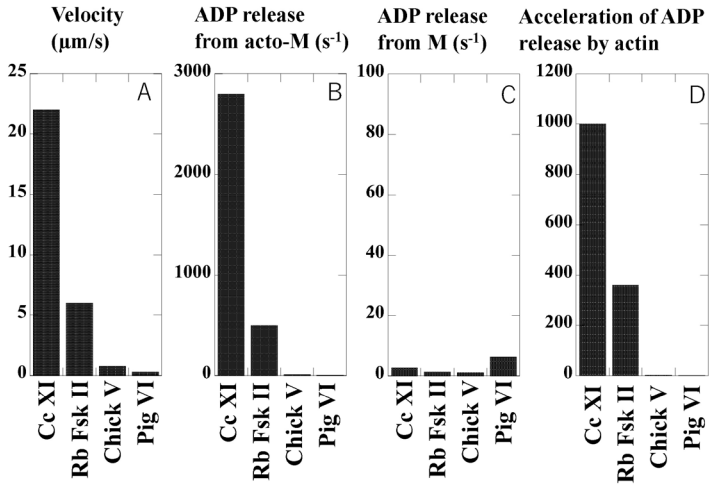

Figure 6 Enzymatic properties of the four classes of myosins: (A) Velocities; (B) ADP dissociation rate from myosin when bound to actin; (C) ADP dissociation rate from myosin when not bound to actin; (D) Acceleration of ADP release by actin. *Cc*XI: *Chara corallina* myosin XI, Rb FsKII: Rabbit fast skeletal muscle myosin II, Chick V: Chicken myosin V, Pig: Pig myosin VI. Data from Ref. [3].

Considering this actin-binding dependent kinetics and the previously mentioned structural analysis, which revealed significant variations in the actin-binding modes of different myosins, it is hypothesized that different classes and subclasses of myosins have different modes of actin binding. These modes would affect the rate at which they promote ADP dissociation, resulting in the observed velocity differences between myosin classes and subclasses. To test this hypothesis, we investigated the effect of mutations in the actin-binding region of myosin on its velocity. Specifically, the fast *Chara* myosin XIs are noted for distinctive features in its actin-binding loop 2 and loop3. Loop 2 in many myosin classes contains numerous positively charged amino acids, mainly lysine, contributing to a net charge ranging from +3 to +6. In contrast, loop 2 of *Chara* myosin XIs possess fewer positively charged amino acids, resulting in a net charge of 0. However, *Chara* myosin XIs compensate for this with more positively charged amino acids in loop 3, thereby preserving their affinity for actin. This was demonstrated by mutant myosin experiments with *Cc*XI as follows.

We mutated loop 2 of *Cc*XI to examine the relationship between loop 2 of *Chara* myosin XIs and the fast motility. When lysine was added to loop 2 of *Cc*XI by genetic mutation to increase the positive charge, the ADP dissociation rate and motility speed decreased. A mutant *Cc*XI with four lysines introduced into loop 2, increasing its charge from 0 to +4 (similar to that of skeletal muscle myosin), had a motility speed that was one-quarter that of wild-type *Cc*XI. This speed was almost equal to that of skeletal muscle myosin, suggesting that the movement speed differences between skeletal muscle myosin and *Cc*XI can be attributed to the differences in the sequence (charge) of loop 2. Conversely, a mutant *Cc*XI in which the charge of loop 2 was reduced from 0 to -2 moved 1.5 times faster than wild-type *Cc*XI. Thus, simply changing the sequence of *Cc*XI's loop 2 resulted in myosin velocities varying by a factor of 6 (i.e., 1.5/0.25) [2].

In many classes of myosins, the positively charged amino acids in loop 2 bind to the negatively charged amino acids at the N-terminus of actin. In contrast, *Chara* myosin XIs have a neutrally charged loop 2 that contributes minimally to actin

binding. Conversely, loop 3 of *Chara* myosin XIs possess more positive charges compared to other classes of myosins, and thus, plays a significant role in binding to actin. Thus, *Chara* myosin XIs compensate for weaker actin binding due to the less positively charged loop 2 with the more positively charged loop 3. The stronger interaction between loop 2 and actin decreases myosin velocity, whereas the interaction between loop 3 and actin does not affect myosin velocity. Thus, the fast movement of *Chara* myosin XIs is primarily due to its unique actin binding pattern, which is facilitated by the specific sequences of loop 2 and loop 3 [2].

*Cb*XI-1, the fastest myosin in the biological world that we have recently cloned, is three times faster than *Cc*XI. Mutation experiments have demonstrated that the velocity difference between *Cb*XI-1 and *Cc*XI is also mainly due to the difference in actin binding. Similar to *Cc*XI, loop 2 of *Cb*XI-1 has a total charge of zero, but it is unique in that it contains an insertion of five glycine residues. This polyglycine insertion is thought to increase the flexibility of loop 2 in *Cb*XI-1. In addition, the CM loop sequence also differs significantly between *Cb*XI-1 and *Cc*XI. A *Cc*XI mutant, in which the loop 2 and CM loop sequences of *Cc*XI were replaced by the corresponding sequences of *Cb*XI-1, showed a 1.5-fold increase in velocity compared to wild-type *Cc*XI. This finding supports the theory that variations in actin-binding sites among myosins not only contribute to velocity diversity across classes and subclasses, but also manifest within individual classes or subclasses [1].

Conclusion and Outlook

We have shown that the secret behind the fast movement of *Chara* myosin XIs resides in the actin-binding site. However, the mechanism by which the diversity of actin-binding sites contributes to the diversity of myosin functions, such as myosin velocity, remains unclear. Upon binding to actin, myosin undergoes a conformational change that closes the cleft at the actin-binding site (Figure 4B). This closure of the cleft induces structural alterations in the nucleotide-binding domain, which promotes the 'actin-activated reaction', leading to accelerated dissociation of ADP and phosphate [29,30]. The manner in which the cleft closes upon actin binding may vary among different myosin classes and subclasses, potentially causing variations in ADP release rates and, consequently, differences in myosin velocities. Recent advances in cryo-electron microscopy have shed light on the binding patterns of various myosins to actin, underscoring the distinctions among them. The relationship between the specific mode of cleft closure and myosin velocity is anticipated to be further clarified in the near future.

Animals can move to find food, but immobile plants store the energy they get from photosynthesis in the form of sugars in their vacuoles. As sugars accumulate in the vacuole, osmotic pressure causes water absorption, leading to cell enlargement - a process known as plant cell growth. While animal cells have an average diameter of about 10 μm, plant cells can grow much larger, with some reaching up to 1 mm. The time required for simple diffusion is proportional to the square of the distance. In a 10 μm animal cell, small molecules such as oxygen and minerals can diffuse throughout the cell in just 0.05 seconds, but it takes 500 seconds for the same molecules to diffuse through a 1 mm plant cell. Plants have therefore developed a system of cytoplasmic streaming to improve diffusion using myosin XI. Because fast cytoplasmic streaming is advantageous for promoting diffusion, myosin XI has evolved to move faster. In addition, the freshwater alga *Chara*, which has large cells with diameters up to 1 mm and lengths up to 10 cm, requires ultra-fast cytoplasmic streaming to maintain such large cells. As a result, myosin XIs of *Chara* would have evolved to achieve extremely high speeds.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

K.I. and T.H. wrote the manuscript and prepared figures.

Data Availability

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request. Full-length nucleotide sequence, atomic coordinate, and structure factor data have been deposited in DNA Data Bank of Japan (DDBJ) and the Protein Data Bank (DDBJ: LC641776, BR001757, BR001749, and BR001750; PDB ID: 7DHW).

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