Energy-Saving Mechanisms in Biological Molecular Machines

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Our skeletal muscles consume much energy when a high tension is maintained for a long time. In contrast, bivalve adductor muscles can maintain high passive tension with little energy expenditure. It has been clarified that its mechanism is based on the molecular interactions of contractile protein molecules in the muscle cells.

1 Introduction

How we make effective use of limited energy is an extremely important issue in today's society. It is the same with living organisms, and a variety of strategies for making effective and efficient use of energy have been implemented throughout the 4 billion year history of evolution. In developing technology for making use of biomolecules such as proteins in building artificial devices, and also in discovering clues for developing a variety of technologies from the energy strategies implemented by natural biomolecules with their long history of evolution, it is highly meaningful to carry out research into their energy-saving mechanisms.

Among all the energy-saving mechanisms of organisms, we have been focusing our research on a mechanism known as "catch" seen in the muscles of bivalves. First we will give a rough outline of what "catch" is, then we will explain our work in developing technology to reconstitute the structure of the "catch" mechanism. At the end, we will also discuss the survival strategy implemented by bivalves during their evolution based on the knowledge we gained from this research and development.

2 The adductor muscle of bivalves consists of multiple parts with different characteristics

When boiling or grilling bivalves such as clams and scallops, their shells open up widely. This is because the hinge joining the two shells is elastic and it causes the shells to open up in the absence of an external force to counteract it. However, the shells usually remain closed most of the time while the shellfish are alive. It is the adductor muscle that closes the shells, and keeps them closed by exerting a constant force[1]. This is a state that would be the equivalent of us holding onto a heavy object all the time. This requires energy, and everyone has no doubt experienced becoming tired from doing this. However, it has been known for a long time that a certain type of muscle found in shellfish consumes next to no energy in doing this[2]. But it has only been in the last 15 years or so that we have begun to understand the mechanism behind this at the biomolecular level.

It can often be seen from looking at the adductor muscle of a live shellfish that it is made up of two parts[3]. Figure 1 shows two species of edible shellfish. On the left is the giant oyster we usually use in our research (scientific name: *Crassostrea gigas*). Its single adductor muscle consists of a yellowish, oval-like portion and a white crescent-shaped part. On the right is a species of scallop, the noble scallop (scientific name: *Mimachlamys nobilis*). Next to the large yellowish and circular portion is a small white part. It is known that the structures of these parts differ at the micrometer level (Fig. 2). The white parts in

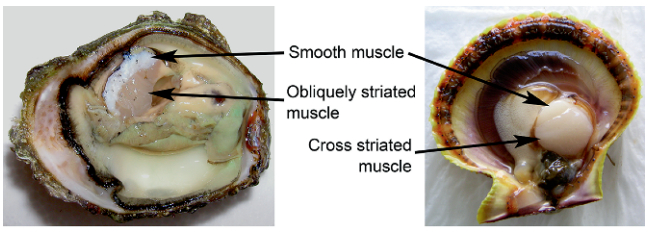

Fig. 1 Adductor muscles of a giant oyster (left) and a noble scallop (right)
The white parts are smooth muscles. The yellow part of the giant oyster is an obliquely striated muscle, while the yellow part of the noble scallop is a cross striated muscle.

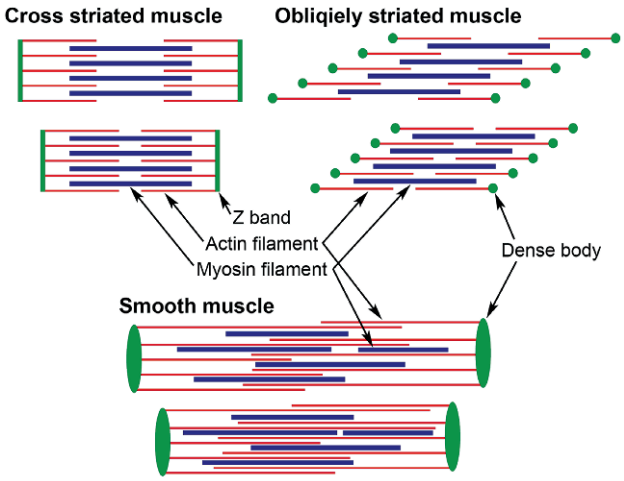

Fig. 2 A schematic diagram of the microstructure of cross striated muscle, obliquely striated muscle and smooth muscle
They all consist of bundles of fibrous protein structures known as myosin filaments (blue) and actin filaments (red), but their arrangements differ. The diagram shows each type of muscle before (above) and after (below) its contraction. The muscles contract due to the myosin filaments and actin filaments sliding along each other. Energy is consumed in this process through the hydrolysis of ATP.

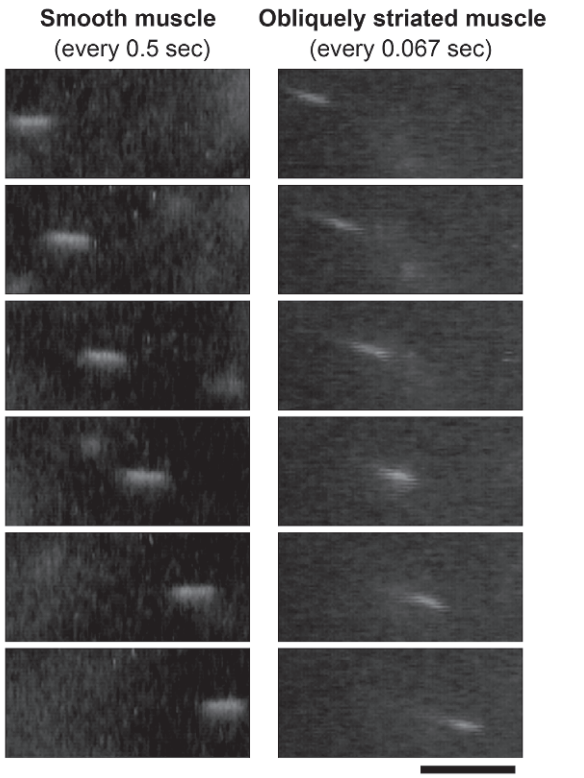

Fig. 3 "*In vitro* reconstitution experiment" of the sliding movements
Sequences of micrographs of actin filaments that slide along myosin filaments prepared from the smooth muscle (left) and the obliquely striated muscle (right) of the giant oyster. Note that the intervals between the two sequences are not the same. Actin filaments slid around five times faster along myosin filaments of obliquely striated muscle than those of smooth muscle. The bar is 5μm.

both species are smooth muscle, and the yellowish part in the giant oyster is obliquely striated muscle, whereas in the noble scallop it is cross striated muscle.

Numerous fibrous protein structures of myosin filaments (Fig. 2: Blue) and actin filaments (Fig. 2: Red) are bundled together within the muscle cells. When the muscle contracts, or in other words when the muscle is in the "activated state," these filaments slide past one another. This requires energy, which is supplied by the chemical reaction of adenosine triphosphate (ATP) being hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate. Proteins extracted from the muscle were reconstituted to recreate this sliding movement, and observed under a light microscope (Fig. 3). Here a fluorescence dye was conjugated to the actin filaments to make them visible for observation. The myosin filaments were fixed onto a glass surface, but they were not visible under fluorescence observations. The actin filaments visualized with the fluorescence dye were seen moving over time. This type of experiment artificially recreating a phenomenon that occurs inside the living body is called an "*in vitro* reconstitution experiment"[4]. "Vitro" means "glass," or it refers in particular to glass equipment used in experiments such as test tubes. The images on the left in Fig. 3 show an actin filament which moved on a myosin filament prepared from the smooth muscle, while the images on the right show an actin filament which moved on a myosin filament prepared from the obliquely striated muscle of a giant oyster. Note that the intervals between images are not the same. Actin filaments moved around five times faster on myosin filaments of obliquely striated muscle than those of smooth muscle[5]. This fact is reflected in the speed at which the muscles contract. Smooth muscle contracts more slowly than obliquely striated muscle. Smooth muscle of scallops also contracts slowly. The cross striated muscle by comparison contracts quickly. It is well known that when scallops are attacked by enemies for example, they flap their shells rapidly to generate a current to swim through the water and escape. They use their large cross striated muscle for this purpose. Oysters also use their obliquely striated muscles to quickly close their shells.

3 The "catch muscle," an energy-efficient-type muscle

Now let us examine what the white part of smooth muscle is used for. As we saw in the experiment shown in

Fig. 3, the sliding movement of the actin filaments due to the myosin filaments in smooth muscle is slow, and the muscle too, contracts slowly. This means that the shells only close slowly. As mentioned earlier, bivalves must overcome the elasticity of their hinges to keep their shells closed as long as they are alive. It is the smooth muscle of shellfish which plays this role. This muscle has been known for a long time as the "catch muscle," and its characteristics have been studied extensively. A "catch" is a type of lock for doors that prevents it from being opened. The "catch muscle" too, is believed to function as a lock that prevents the shells from opening completely. In its locked state, it is said to be in a "catch state," and it is in this state that the "catch muscle" is able to "hold on" using far less energy as an energy-efficient-type muscle, than the muscles in our hands and legs would use when holding onto something.

The catch muscles enter the catch state just after their active contractions. With our hands and legs, the muscles become soft the moment we relax after contracting them, but in the case of the catch muscles they enter the "catch state" to maintain their firmness. However, it is not convenient to maintain this state forever. A lot of bivalves feed on plankton, and they must draw water containing plankton into their bodies. Of course, they must take oxygen from the water, too. They must leave their shells open slightly to take in water containing plankton and oxygen from their surroundings, and discharge waste from their bodies.

They use phosphorylation-dephosphorylation (Fig. 4) mechanism of proteins commonly found among lives to switch between the catch and relaxed states. The protein involved in controlling the catch mechanism is called twitchin, and it is known to be associated with myosin filaments. When particular hydroxyl groups on this protein become phosphorylated by a protein kinase, the catch state is released and the muscle relaxes[6]. It is a protein phosphatase that removes the phosphate groups to return them to the original hydroxyl groups[7].

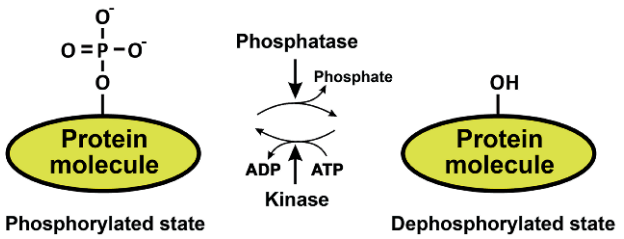

Fig. 4 The phosphorylation and dephosphorylation of a protein molecule

The ATP and enzymes act to form a reversible covalent bond between phosphoric acid and a specific part of the protein molecule. This system, making use of these two different states to control a variety of mechanisms, is found widely in the cells of organisms.

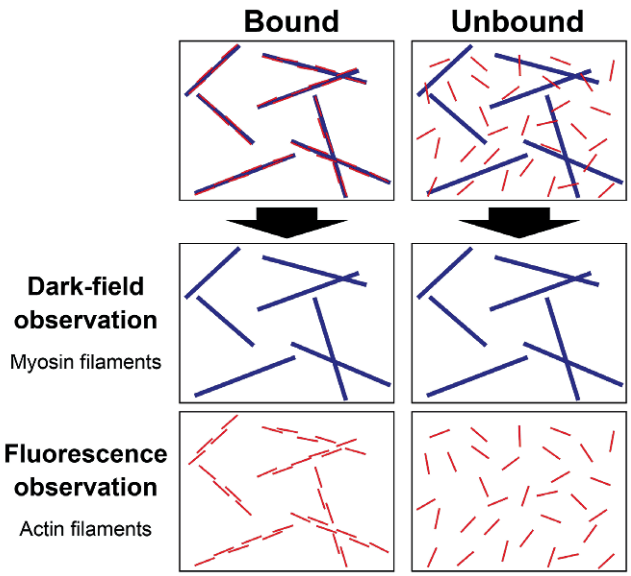

Fig. 5 An experimental method for distinguishing myosin filaments (blue) and actin filaments (red) in the bound state (left) and unbound state (right) under a light microscope

Myosin and actin filaments are visualized by the dark-field and fluorescence illuminations, respectively. If the two types of filaments are bound, the images taken by the two methods should match almost perfectly. If they are unbound, the images will not match at all.

4 *In vitro* reconstitution of the catch state

It has already been stated that a sliding movement occurs between the myosin filaments and actin filaments when muscles contract or enter the "activated state." So let us take a look now at what happens to these filaments during the "catch" and "relaxed" states. We succeeded in reconstituting these states *in vitro*, just like when we observed the sliding movement of filaments in the muscle in the contracting state. As a result we discovered that when the muscle maintained a state of high tension in the "catch state," the actin filaments bound to the myosin filaments, while in the "relaxed state" the filaments rarely bound[8].

Figure 5 schematically shows the experimental method we used to distinguish this "bound state" and "unbound state"[9]. It is known that myosin filaments are considerably thicker than actin filaments so they scatter visible light to a greater extent. Therefore it was possible to observe only myosin filaments under the dark-field light microscopic observation that was useful for observation of the scattered light. On the other hand, actin filaments were conjugated

with fluorescence dye as in the Fig. 3 experiment. Only actin filaments were observed under the fluorescence illumination. If the two types of filaments were bound, observation of the same field of view through the dark-field and fluorescence illuminations would reveal that the filaments matched almost perfectly. If the filaments were unbound, they would not match.

In the experiment shown in Fig. 3, the actin filaments moved on the myosin filaments. While this happened, the calcium ion concentration was high in the solution that the filaments were bathed in. In general, when muscles contract the calcium ion concentration within cells increases and this acts as a signal for the filaments to move. When the calcium ion concentration within cells decreases, the muscles in our hands and legs relax, but in the case of the catch muscles they can either relax or enter the catch state. What controls this is the phosphorylation-dephosphorylation of the protein, twitchin, mentioned earlier. When twitchin is phosphorylated, it enters the "relaxed state," and when it is dephosphorylated it enters the "catch state," under low calcium ion concentrations.

So let us take a look at our experiment now (Fig. 6). We maintained low calcium ion concentrations. In the absence of twitchin almost no actin filaments bound to myosin filaments (Fig. 6 right: "Completely relaxed state"). By contrast, in the presence of dephosphorylated twitchin, the actin filaments bound strongly to the myosin filaments to form thick bundles (Fig. 6 left: "Catch state"). This state just corresponds to the catch state that enables the catch muscles to maintain high tension. And the actin filaments rarely bound to the myosin filaments in the presence of phosphorylated twitchin (Fig. 6 center: "Relaxed state").

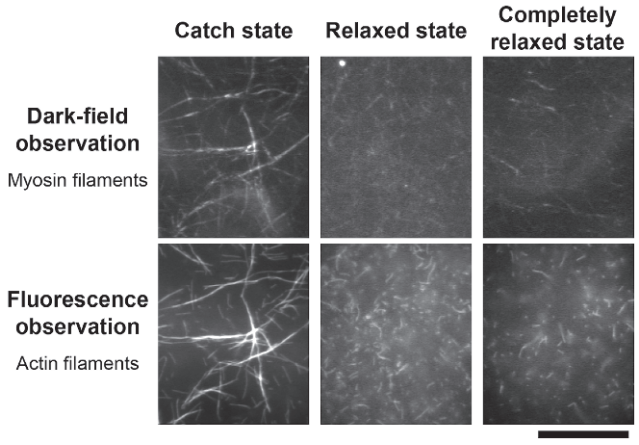

Fig. 6 Experiment to reconstitute the catch state *in vitro* using myosin and twitchin prepared from the smooth muscle of a giant oyster
The bar is 20 μm

There were a few actin filaments bound to myosin filaments, but this might have been due to incomplete phosphorylation of twitchin in the experiment.

5 A catch state in muscles other than the "catch muscle"

The muscles known as the "catch muscles" for many years are the smooth parts of bivalve adductors. Other muscles were not considered to be "catch muscles." In our research at the molecular level, we have discovered that the catch state can be reconstituted by using only myosin filaments, actin filaments, twitchin, and enzymes that control the phosphorylation state of twitchin. It is known that myosin and actin filaments are not limited to muscle cells, but they are found widely in most eukaryotic cells. Twitchin is a protein originally found in nematodes[10], and similar proteins are found also in vertebrates including human. Protein kinases and phosphatases that change the phosphorylation state of twitchin are also found widely in eukaryotic cells. These facts give rise to a question whether the "catch" mechanism is not unique to the smooth muscles of bivalves, but exists in the tissues of other animals.

As a first step toward answering this question, we decided to carry out an experiment on the obliquely striated muscle of giant oysters and the cross striated muscle of noble scallops. Twitchin is found in these muscles too, although in smaller quantities than in smooth muscles. Myosin and twitchin were extracted and purified from these muscles, and the same experiment as shown in Fig. 6 was carried out, which achieved the same results[5]. It is presumably because these muscle cells contain little twitchin that they do not show a clear "catch state" of high tension as the smooth muscles. But they may be able to maintain tension to some extent after their active contractions.

If the striated muscles of the adductors were to contain larger amounts of twitchin, they could be in a catch state as the smooth muscles. If this were the case, they would be able to use their striated muscles to keep their shells closed, and they would not need smooth muscles. But the actual evolution of bivalves did not advance in this direction, and instead opted to equip them with smooth muscles separated from their striated muscles. We asked ourselves why this happened in the long history of bivalves.

6 Energy consumption during the catch state

Research into the oxygen consumption of the catch muscles has revealed that energy consumption in the catch state is very low. However, their experimental methods have been nothing more than the measurement of total energy consumption by the various elements that exist within muscle cells. Furthermore, the amount of oxygen consumed at a particular point in time is generally not necessarily equivalent to energy consumption at the same point in time, because an "oxygen debt" may be created and made up for later. To be more precise it can also be argued that the amount of oxygen required for the same amount of energy will differ depending on whether the source of energy is glucose, fatty acid, etc., resulting in an error.

We used only proteins extracted and purified from muscles, and succeeded in reconstituting the "activated state" in which the filaments slide (Fig. 3), the "catch state" in which the filaments maintain high tension (Fig. 6 left), the "relaxed state" in which the catch is relaxed (Fig. 6 center), and the "completely relaxed state" in which the protein twitchin needed to create the catch state is absent (Fig. 6 right) *in vitro*. The energy used in these reconstituted systems does not come from the use of oxygen, but it comes solely from the hydrolysis of ATP carried out directly by the motor protein, myosin. Therefore we can exclude various factors that give rise to the kind of errors mentioned above, making it possible to assess energy consumption under the different conditions. We summarized the measured values of energy consumptions taken from the purified systems prepared from the smooth muscle and obliquely striated muscle of giant oysters and compared them in Table 1. The speeds of the sliding movements such as shown in Fig. 3 are also included.

In the "activated state" the sliding speed of the obliquely striated muscle was roughly five times the speed of the smooth muscle, but energy consumption was as much as around 10 times more. Subtle energy consumption was observed in the "completely relaxed state," but it was only around one thousandth of what it was in the "activated state" in each muscle. It should be noted that a certain amount of energy was consumed in the "catch state" too. This was around one hundredth of the energy consumed in the "activated state" in each muscle, and the obliquely striated muscle consumed 3–4 times as much energy as the smooth muscle. Almost no energy was consumed when twitchin was phosphorylated in the "relaxed state." These facts indicate that energy is required even in maintaining the catch, and that the obliquely striated muscle requires more energy than the smooth muscle in the catch state[11].

Table 1 Comparison of sliding speeds and energy consumptions (ATP hydrolysis rates) under a variety of conditions reconstituted from myosin filaments and twitchin prepared from the smooth muscle and obliquely striated muscle of a giant oyster

	Smooth muscle	Obliquely striated muscle
Speed of sliding movments in the activated state*	3.3±0.3 μm s^{-1}	16.4±2.0 μm s^{-1}
Energy consumption in the activated state**	0.82±0.16 s^{-1}	9.5±2.1 s^{-1}
Energy consumption in the catch state**	0.013±0.001 s^{-1}	0.041±0.005 s^{-1}
Energy consumption in the relaxed state**	0.0036±0.0007 s^{-1}	0.011±0.002 s^{-1}
Energy consumption in the completely relaxed state**	0.0012±0.0001 s^{-1}	0.0047±0.0007 s^{-1}

*: Mean ± standard deviation. Tsutsui et al. (2007)
**: The speed at which one myosin molecule hydrolyzes ATP. Mean ± standard error. Yamada et al. (2013)

Perhaps herein lies the answer to the reason why bivalves evolved the smooth muscles as the "catch muscles," separated from the striated muscles. They require less energy to use the smooth muscles rather than the obliquely striated muscles or cross striated muscles to hold the shells closed in the catch state. Perhaps this was their energy-saving strategy in the long history of their evolution.

7 Conclusions

As indicated by the expression, "biological diversity," there are a variety of organisms on Earth each with their own strategies for surviving. The energy-saving strategy implemented by bivalves in their adductor muscles is an example. Research into these strategies is highly important for utilizing biomaterials in technology, and also for developing all kinds of technology based on the survival strategies of life. However, not everything has yet been elucidated on the catch mechanism of bivalves, let alone the strategies implemented by the great variety of organisms. These are the reasons why we must continue such kind of fundamental research as we have reported on in this paper.

References

1 J. Lowy and B. M. Millman, "The contractile mechanism of the anterior byssus retractor muscle of *Mytilus edulis*," Phil. Trans. B, Vol. 246, No. 728, pp. 105–148, Jan. 1963.

2 F. Baguet and J. M. Gillis, "Energy cost of tonic contraction in a lamellibranch catch muscle," J. Physiol., Vol. 198, No. 1, pp. 127–143, Sep. 1968.

3 J. Bowden, "The structure and innervation of lamellibranch muscle," Intl. Rev. Cytol., Vol. 7. pp. 295–335, 1958.

4 S. J. Kron and J. A. Spudich, "Fluorescent actin filaments move on myosin fixed to a glass surface," Proc. Natl. Acad. Sci. USA, Vol. 83, No. 17, pp. 6272–6276, Sep. 1986.

5 Y. Tsutsui, M. Yoshio, K. Oiwa, and A. Yamada, "Striated muscle twitchin of bivalves has "catchability", the ability to bind thick filaments tightly to thin filaments, representing the catch state," J. Mol. Biol., Vol. 365, No. 2, pp. 325–332, Jan. 2007.

6 M. J. Siegman, D. Funabara, S. Kinoshita, S. Watabe, D. J. Hartshorne, and T. M. Butler, "Phosphorylation of a twitchin-related protein controls catch and calcium sensitivity of force production in invertebrate smooth muscle," Proc. Natl. Acad. Sci. USA, Vol. 95, No. 9, pp. 5383–5388, April 1998.

7 L. Castellani and C. Cohen, "A calcineurin-like phosphatase is required for catch contraction," FEBS Lett., Vol. 309, No. 3, pp. 321–326, Sep. 1992.

8 A. Yamada, M. Yoshio, H. Kojima, and K. Oiwa, "An *in vitro* assay reveals essential protein components for the "catch" state of invertebrate smooth muscle," Proc. Natl. Acad. Sci. USA, Vol. 98, No. 12, pp. 6635–6640, Jun. 2001.

9 Y. Tsutsui, M. Yoshio, K. Oiwa, and A. Yamada, "Twitchin purified from molluscan catch muscles regulates interactions between actin and myosin filaments at rest in a phosphorylation-dependent manner," J. Muscle Res. Cell Motil., Vol. 26, No. 6–8, pp. 461–465, Dec. 2005.

10 G. M. Benian, J. E. Kiff, N. Neckelmann, D. G. Moerman, and R. H. Waterston, "Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans*," Nature, Vol. 342, No. 6245, pp.45–50, Nov. 1989.

11 A. Yamada, M. Yoshio, and K. Oiwa, "Myosin Mg-ATPase of molluscan muscles is slightly activated by F-actin under catch state in vitro," J. Muscle Res. Cell Motil. Vol. 34, No. 2, pp. 115–123, May 2013.

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