Cytoplasmic streaming is unique to and important for plant cells because plant cells, due to their large size, cannot rely on simple diffusion for the transportation and distribution of essential molecules throughout the cell. However, the energy used by plant cells to generate cytoplasmic streaming cannot rely on simple diffusion for the transportation and distribution of essential molecules throughout the cell. It is known that this cytoplasmic streaming is generated by the sliding movement of a myosin (class XI) along the actin cables fixed on the surface of chloroplasts lining the cytosolic face of the cell membrane (Kamiya and Kuroda 1956, Kamitsubo 1966, Nagai and Rebhun 1966, Kachar and Reese 1988, Yamamoto et al. 1994, Kashiyama et al. 2000). Hydrodynamic considerations suggested that movement of myosin alone is not enough to generate the streaming. Myosin should move with its tail attached to the endoplasmic reticulum meshwork to produce bulk flow of water (Nothnagel and Webb 1982). Recently, it was found that the myosin responsible for the streaming in Chara corallina has very high ATPase activity and the activity is 10 times higher than that of skeletal muscle myosin (Ito et al. 2003). The activity of this myosin is so high that, if its concentration in the cell were high, a large amount of ATP would be consumed in the Chara cell by the cytoplasmic streaming. If this is the case, the Chara cell can be a good model to estimate the energy consumed by cytoplasmic streaming in plant cells. We therefore estimated both the amount of this myosin in the Chara cell by quantitative immunoblot and the supply of ATP by metabolic reaction by measuring dark respiration. It was found that the concentration of myosin in the Chara cell was considerably high but the supply of ATP by metabolic reaction was not enough to support the high activity of myosin, if all myosin molecules were fully activated. Since the cytoplasmic streaming of the Chara cell does not stop even at night, we speculate that only a limited amount of myosin interacts with actin cables and generates the force for the cytoplasmic streaming. This idea was supported by the calculations of energy required to generate the cytoplasmic streaming in the Chara cell.

We first estimated the amount of myosin in C. corallina internodal cells by quantitative immunoblot as described below. The sum of the length of the internodal cells used was 2,480 mm. We obtained 356 μL of SDS sample from them. A 1 μL aliquot of the sample was subjected to SDS–PAGE and Chara myosin was detected by immunoblot and the supply of ATP by metabolic reaction by measuring dark respiration. It was found that the concentration of myosin in the Chara cell was considerably high but the supply of ATP by metabolic reaction was not enough to support the high activity of myosin, if all myosin molecules were fully activated. Since the cytoplasmic streaming of the Chara cell does not stop even at night, we speculate that only a limited amount of myosin interacts with actin cables and generates the force for the cytoplasmic streaming. This idea was supported by the calculations of energy required to generate the cytoplasmic streaming in the Chara cell.

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Energy required for cytoplasmic streaming in Chara

The amount of myosin in the Chara cell estimated by quantitative immunoblot. The standard curve was drawn from the extent of color development of a known amount of globular tail domain expressed molecular biologically (open circle). A typical result of the immunoblot is shown in the inserted photo (left four lanes). The bars indicate the SD of four measurements. Color development at the position of Chara myosin heavy chain was also quantitated (filled circle) under the same conditions. A typical result is also shown in the inserted photo (right end lane).

Applied Tail Domain (ng) vs. Color Development (arbitrary unit)

Fig. 1

The required ATP is thus 0.311 l s−1 cm−2. The concentration of ATP in the Chara cell is around 5 mM at best and it would be depleted in less than a couple of minutes if a considerable amount of ATP were not supplied by metabolic reaction and photosynthesis. Actually, when we mixed 50 nM recombinant Chara myosin with 4 mM ATP in the presence of 1 mg ml−1 F-actin, the ATP decreased in a linear manner to zero in 400 s. This means that the affinity of Chara myosin for ATP is very high and the resultant ADP up to 4 mM does not affect the ATPase activity of Chara myosin. Since cytoplasmic streaming does not stop even at night, it can be said either that the amount of ATP supplied by metabolic reaction is very large in the Chara cell or that only a limited amount of myosin is activated by actin in the cell.

To estimate the supply of ATP at night, we measured dark respiration of isolated mature C. corallina internodal cells using an oxygen electrode at 27°C. The decrease in oxygen concentration was followed for 2–2 h in the dark. The rate of decrease was constant during that period, suggesting that the measured value was not a transient one. The value was 0.60 ± 0.15 mg O2 g DW−1 h−1 (n = 4). Since the ratio of dry weight to fresh weight of Chara cells is 0.077 (T. Tsuchiya and K. Yamamoto unpublished observation), a gram of dry weight corresponds to a cell volume of about 13 ml. If we assume that the P/O ratio is 3 and the volume of cytoplasm is 5% of the total cell volume, ATP supplied by metabolic reaction is 48 μmol s−1 cm−2 at 27°C. This value is comparable with those for various characean algae measured by Sorrell et al. (2001) at 15°C (11–32 μmol). The result suggests both that ATP supplied by metabolic reaction is not enough to support the fully activated Chara myosin and that not all myosin molecules are working in the cell.

The motive force for the cytoplasmic streaming was estimated from the force applied to stop it by using either centrifugation (Kamiya and Kuroda 1958) or vacuolar perfusion (Tazawa 1968, Donaldson 1972). Kamiya and Kuroda (1973) also measured the velocity gradient in the cytoplasm of a compressed Nitella cell and calculated the motive force. The estimated values were 0.1–0.4 Pa (N m−2). The energy required to generate cytoplasmic streaming can be calculated from these values. We imagine an internodal cell having a length and diameter of 10 cm and 0.1 cm (1 mm), respectively. The total inner surface of this cylindrical cell is 3.16 cm2. The total force generated at the interface between the cytoplasm and the surface of chloroplasts where actin cables are fixed is 9.48 × 10−5 N (we adopted 0.3 Pa for the motive force). Cytoplasm moves about 100 μm (10−3 m) in 1 s. The energy required for this movement is 9.48 × 10−9 J. When 1 mol of ATP is hydrolyzed to ADP and Pi, the liberated energy is 30.5 kJ. The required ATP is thus 0.311 × 10−12 mol s−1. If we assume that the thickness of cytoplasm is 10 μm,
the volume of the cytoplasm is $3.13 \times 10^{-6}$ l. ATP consumed by the cytoplasmic streaming is, therefore, $0.994 \times 10^{-1}$ mol s$^{-1}$ l$^{-1}$. This value is one- to two-thousandth those estimated above assuming that all myosin molecules are fully activated (100 $\mu$mol s$^{-1}$) and from dark respiration (48 $\mu$mol s$^{-1}$).

Because the difference between the estimated values was so great, we tested the validity of the estimated value of the motive force by calculating the mechanical shear force at the interface between the moving cytoplasm and the surface of stationary chloroplasts. It is known that the viscosity of characean cytoplasm is very high so that the cytoplasmic layer moves as a unit in a living cell (Kamiya and Kuroda 1963). We assumed that the Chara cytoplasm is a thin rigid plate with a thickness of 10 $\mu$m moving at a velocity of 100 $\mu$m s$^{-1}$. The force $F$ required to keep such a plate with the unit area moving at a velocity of $v$ relative to the other parallel plate separated by a distance $z$ can be written as follows:

$$F = \eta d v / d z$$

where $\eta$ is the viscosity of a solution filling the space between the two plates. Because the viscosity of Chara cytosol (not cytoplasm) is not known, we estimated it experimentally. We thought that the viscosity of the cytosol was similar to that of a saline solution containing a high concentration of protein and measured the viscosity of 10 mg ml$^{-1}$ bovine serum albumin dissolved in phosphate-buffered saline using an Ostwald viscometer at 20 $^\circ$C. The relative density $\rho$ of solutions was determined by weighing the same volume of solutions sucked with the same liquid dispenser. Flow time $t$ and relative density are shown in Table 1. The viscosity of the albumin solution was calculated as follows:

$$\eta_{\text{sample}} = \eta_{\text{water}} \frac{\rho_{\text{sample}} \times t_{\text{sample}}}{\rho_{\text{water}} \times t_{\text{water}}}$$

The viscosity of water at 20 $^\circ$C is $1 \times 10^{-3}$ Pa s. We, therefore, assumed the viscosity of Chara cytosol to be $1.2 \times 10^{-2}$ Pa s. Even if the protein concentration in Chara cytosol were much higher than 10 mg ml$^{-1}$, the viscosity would not exceed $1.5 \times 10^{-3}$ Pa s. We estimated the separation between the cytoplasm and the surface of chloroplast as follows (Fig. 2). There are 3-4 actin cables on one chloroplast and their diameter is 100–200 nm (Kamitsubo 1966, Nagai and Rebhun 1967). The size of Chara myosin observed by electron microscopy is about 100 nm (Yamamoto et al. 1995). Chara cytoplasm contains a meshwork of endoplasmic reticulum membrane and the end of the mesh protrudes about 200 nm from the rest by being pulled by myosin (Kachar and Reese 1988).

![Fig. 2 Schematic illustration of the cross-section of Chara internodal cell showing structural elements at the interface between moving cytoplasm and stationary chloroplasts lining the cytosolic face of the cell membrane.](image)

The separation thus estimated was 400–500 nm (0.4–0.5 $\mu$m). The force calculated from these values was 0.24–0.3 Pa and these values were in good agreement with the values estimated experimentally (Kamiya and Kuroda 1958, Tazawa 1968, Donaldson 1972, Kamiya and Kuroda 1973).

Our calculations suggested that the energy required for the cytoplasmic streaming is very low even in characean algal cells that display very fast cytoplasmic streaming all around the cell. The energy required to generate cytoplasmic streaming was about 0.2% of dark respiration. This is probably the reason why the energy for cytoplasmic streaming has not been considered seriously as a component of the maintenance energy (respiration) in plants.

Our results also suggested that myosin was abundant in the Chara cell, but only a limited number of myosin molecules were actively generating force for the cytoplasmic streaming. Because Chara myosin moves very fast, it may not be able to convert the free energy liberated by the hydrolysis of ATP efficiently into mechanical force. Even if we assume that the efficiency is 10%, only one-hundredth of the total myosin is enough to generate the force of cytoplasmic streaming in Chara. Myosin molecules are

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**Table 1** Viscosity of bovine serum albumin solution

<table>
<thead>
<tr>
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<th>Flow time$^a$</th>
<th>Relative density$^a$</th>
<th>Relative viscosity</th>
</tr>
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<tbody>
<tr>
<td>Water</td>
<td>76.2</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>79.6</td>
<td>1.016</td>
<td>1.063</td>
</tr>
<tr>
<td>Bovine serum albumin solution</td>
<td>83.0</td>
<td>1.024</td>
<td>1.115</td>
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$^a$Values are the average of three measurements.
probably distributed on various kinds of internal membrane but concentrated mostly on the meshwork of endoplasmic reticulum membrane near the actin cables because of its affinity for actin. Active rotation of detached chloroplasts was sometimes observed in the flowing cytoplasm of Chara or Nitella (Kamitsubo and Kikuyama 1992). This can be explained by the interaction between such myosin and a fragment of actin cable attached on the chloroplast surface. Non-specific attachment of Chara myosin to the internal membrane is due to its affinity for acidic phospholipids such as phosphatidylserine (Yamamoto et al. 1995) probably through its globular tail domain. The situation is schematically illustrated in Fig. 3a. Immunofluorescence microscopic observation of Chara cells also revealed that most of the fluorescence antibodies against myosin gathered around actin cables (Fig. 3b). The amount of myosin attached to the other internal membrane may not be so large and we cannot see it clearly due to the strong autofluorescence of chloroplasts. Occasional strongly fluorescent spots on actin cables probably represent artifactual myosin stack due to fixation. Glutaraldehyde cannot stop all myosin molecules at once. If one myosin molecule stops moving due to the glutaraldehyde fixation, it will disturb other myosin molecules following the same actin track. This small stack will affect the movement of myosin molecules on the next actin filament in the same cable.

An intriguing question is why there are so many myosin molecules in the Chara cell as shown in this study. One reason for this might be the instability of Chara myosin. Chara myosin can move very fast but is, at the same time, very unstable. We have observed that Chara myosin loses its activity very easily during and after the purification process (Yamamoto et al. 1994). Because of this instability, Chara myosin must be recruited frequently from a large store. If this is the case, there must be some regulatory mechanism to suppress unnecessary interaction between Chara myosin and actin to save both ATP and myosin molecules. The other reason might derive from kinetics. Because cytoplasmic streaming in Chara cells is very fast, most of the myosin molecules may fail to grab actin cables. If the probability of this attachment is very low (1–2%) at this streaming velocity, there has to be 50–100 times more myosin molecules than those required for the streaming. A similar decrease in the attachment probability between myosin and actin was suggested to occur in rapidly shortening skeletal muscle (Huxley 1957). Further investigations are required to understand the regulatory mechanism of myosin–actin interaction and the turnover of myosin in Chara.

**Materials and Methods**

Expression of the globular tail domain of Chara myosin and its purification was done as described by Awata et al. (2003). Briefly, Gly1639 to Ala2182 of Chara myosin was connected to glutathione S-transferase and the construct was expressed in High Five cells and purified using a glutathione-Sepharose column.

The quantitative immunoblot was performed as follows. Antibody against the globular tail domain of Chara myosin was raised in rabbit and purified as described previously (Awata et al. 2003). Crude cytoplasm of C. corallina internodal cells was obtained by squeezing the cell after removing the vacuolar sap by internal perfusion with a solution containing protease inhibitors (Yamamoto et al. 1994). The crude cytoplasm was immediately boiled with SDS and electrophoresed on an 8% polyacrylamide gel. Several known amounts of purified globular tail domain of Chara myosin were also electrophoresed on a separate gel (12%). Proteins were transferred to nitrocellulose membrane under different conditions because of the difference in the transfer efficiency. To examine conditions for the transfer and to estimate the transfer efficiency of Chara myosin heavy chain, we used rabbit skeletal muscle myosin heavy chain because its molecular weight is close to that of Chara myosin and we could easily obtain the myosin in large quantities. Exactly the same amount of muscle...
myosin (1 μg) was electrophoresed on the same gel in separate lanes and the gel was cut into two pieces after electrophoresis in between the two lanes. One was stained with Coomassie brilliant blue and the other was used to test the condition for transfer. The latter gel was also stained with Coomassie brilliant blue after the transfer to examine the amount of myosin left in the gel. The amount of myosin transferred to the membrane was examined by Amido Black staining. We used two layers of membrane to examine the amount of myosin passing through the first membrane. The best result was obtained when myosin was electrophoresed in an 8% polyacrylamide gel and transferred to the membrane at 150 mA for 2 h in 25 mM Tris, 192 mM glycine and 0.02% SDS. The transfer efficiency was 87% as judged from the amount of myosin left in the gel before and after transfer, and there was no indication of myosin passing through the first membrane. Similar experiments suggested that electrophoresis in a 12% polyacrylamide gel and transfer to the membrane at 150 mA for 1 h in 25 mM Tris, 192 mM glycine and 0.02% SDS gave the best result for the globular tail domain of *Chara* myosin. The transfer efficiency was 100%. The membrane was treated with the primary antibody against the globular tail domain of *Chara* myosin and then the primary antibody was recognized by the anti-rabbit IgG conjugated with alkaline phosphatase. The membrane holding proteins in the *Chara* cytoplasm and that holding various known amounts of the globular tail domain of *Chara* myosin were put together in one container and color development by alkaline phosphatase activity was carried out for the same period of time. The color development was quantitated by densitometry and the standard curve was drawn from the values for the globular tail domain. The amount of *Chara* myosin was determined from the standard curve assuming that the globular tail domain of *Chara* myosin reacts with the antibody in the same manner as that expressed molecular biologically. The difference in the molecular weight and the transfer efficiency was included in the calculation to estimate the amount of *Chara* myosin.

Dark respiration was measured as follows. Internodal cells of *C. corallina* (3–4 cm long) were cut and the vacuole was perfused with a solution containing 80 mM sucrose, 70 mM KCl, 5 mM MgCl2, 5 mM EGTA and 10 mM MOPS buffer pH 7.0 (solution A). Cells were perfused with a solution containing 0.2 M glutaraldehyde and 4 mM MOPS buffer pH 7.0. Cells were exposed to microwave irradiation using a commercial microwave oven at 10 min. Then cells were treated with antibody against the globular tail domain of *Chara* myosin dissolved in the blocking solution and left for 10 min. Then cells were treated with antibody against the globular tail domain of *Chara* myosin dissolved in the blocking solution for 30 min. These cells were washed with solution A and then treated with fluorescein-labeled antibody against rabbit IgG (Promega, USA) for 30 min. Cells were cut into small segments (1–2 mm in length) and immersed in 50% glycerol containing 0.5% p-phenylenediamine as antifade solution. In this glycerol solution, cylindrical segments were cut to make single layer preparations with the cytosolic face up. The sample was observed and photographed by a Nikon TMD inverted microscope equipped with epifluorescence optics.

### References


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