Intracellular Na\(^+\) Kinetically Interferes with the Rotation of the Na\(^+\)-driven Flagellar Motors of Vibrio alginolyticus*

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To understand the mechanism of Na\(^+\) movement through the force-generating units of the Na\(^+\)-driven flagellar motors of Vibrio alginolyticus, the effect of intracellular Na\(^+\) concentration on motor rotation was investigated. Control cells containing about 50 mM Na\(^+\) showed good motility even at 10 mM Na\(^+\) in the medium, i.e. in the absence of an inwardly directed Na\(^+\) gradient. In contrast, Na\(^+\)-loaded cells containing about 400 mM Na\(^+\) showed very poor motility at 500 mM Na\(^+\) in the medium, i.e. even in the presence of an inwardly directed Na\(^+\) gradient. The membrane potential of the cells, which is a major driving force for the motor under these conditions, was not detectably altered, and consistently with this, Na\(^+\)-coupled sucrose transport was only partly reduced in the Na\(^+\)-loaded cells. Motility of the Na\(^+\)-loaded cells was restored by decreasing the intracellular Na\(^+\) concentration, and the rate of restoration of motility correlated with the rate of the Na\(^+\) decrease. These results indicate that the absolute concentration of the intracellular Na\(^+\) is a determinant of the rotation rate of the Na\(^+\)-driven flagellar motors of V. alginolyticus. A simple explanation for this phenomenon is that the force-generating unit of the motor has an intracellular Na\(^+\)-binding site, at which the intracellular Na\(^+\) kinetically interferes with the rate of Na\(^+\) influx for motor rotation.

Bacterial flagellar motors rotate helical flagellar filaments to give the propulsive force to the cell body. The motors are embedded in the cytoplasmic membrane, and the energy source for these motors is the electrochemical potential gradient of a specific ion across the membrane, the ion-motive force, which is composed of the membrane potential and the chemical gradient of the ion (for reviews, see Refs. 1 and 2). Thus, the bacterial flagellar motor is a molecular machine, which converts the energy of ion flux into mechanical work.

Based on the species of the coupling ion, the flagellar motors are classified into two types; one is the H\(^+\)-driven motors which converts the energy of ion flux into mechanical work. Thus, the bacterial flagellar motor is a molecular machine, and its Na\(^+\) pump-defective mutant, Nap1 (16), were grown at 37 °C with shaking in synthetic medium (pH 7.5) as described previously (17). At the late log phase of growth, cells were harvested by centrifugation at 8000 × g for 5 min at room temperature, re-suspended in the same medium as a dense cell suspension, and kept on ice until use.

For some experiments, E. coli AB1200, a smooth-swimming mutant (18), was used, and the cells were grown in tryptone broth containing 0.5% glycerol as described previously (19).

Preparation of Cells Loaded with Specified Cations—Specified cations were loaded to the cells as described previously (15). Briefly, the harvested cells of V. alginolyticus were resuspended in 50 mM diethanolamine-HCl (pH 8.5) containing 10 mM MgCl\(_2\) and 0.4 M desired cation as a chloride salt, incubated at 25 °C for 10 min, and harvested by centrifugation. This treatment was repeated once. Then, the cells were similarly treated twice at 25 °C for 5 min with 50 mM HEPES1 (pH 7.0) containing 10 mM MgCl\(_2\) and 0.4 M desired cation. After being concentrated as a dense cell suspension in the same medium, potential and the chemical gradient of the respective coupling ion (6, 9–11). For motor rotation, the role of both the extracellular and intracellular concentrations of the coupling ion has been considered to be simply to determine the size of the ion gradient. This has been thought to be a characteristic property of the energy coupling mechanism of these motors.

Recently, however, Sugiyama et al. (12) reported that the Na\(^+\)-driven flagellar motors of alkalophilic Bacillus were specifically inhibited by amiloride, a potent inhibitor for the Na\(^+\) channels in animal cells (13, 14). Kinetic analysis revealed that amiloride inhibited motility not by decreasing the Na\(^+\) flux through the motor by competing with Na\(^+\) in the medium (2, 12), indicating the presence of a Na\(^+\)-binding site at the external face of the force-generating unit of the motor. Then, we need to consider that the motor rotation should be affected by the affinity of this site for Na\(^+\), namely by the absolute concentration of Na\(^+\) in the medium. The situation would be even more complicated if the force-generating unit has another Na\(^+\)-binding site on the cytoplasmic side.

To clarify whether or not the force-generating unit of the Na\(^+\)-driven motor has an intracellular Na\(^+\)-binding site, we have examined the effect of the intracellular concentration of Na\(^+\) on motor rotation. For this, we used Vibrio alginolyticus because a method for changing the intracellular cations has been established in this bacterial species (15). In this report, we describe that intracellular Na\(^+\) kinetically inhibits the motor rotation. This implies that the force-generating unit of the Na\(^+\)-driven motor has an intracellular Na\(^+\)-binding site.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—V. alginolyticus 138-2 and its Na\(^+\) pump-defective mutant, Nap1 (16), were grown at 37 °C with shaking in synthetic medium (pH 7.5) as described previously (17). At the late log phase of growth, cells were harvested by centrifugation at 8000 × g for 5 min at room temperature, re-suspended in the same medium as a dense cell suspension, and kept on ice until use.

The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; AIB, α-aminoisobutyrate; TFMF\(^-\), triphenylmethylphosphonium ion; Tricine, N-[2-hydroxy-1,1-bis (hydroxymethyl)ethyl]glycine.

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the cells were kept on ice until use. For E. coli, the concentration of desired cation for loading was 0.14 M and MgCl₂ was omitted. 

Measurement of Motility—For the measurement of motility in V. alginolyticus, motility medium consisting of 50 mM HEPES (pH 7.0), 5 mM glucose, and 0.4 M specified salts was used. In most cases, the pH of the medium was adjusted by NaOH. However, when the Na⁺ concentration in the medium was varied, the pH of the medium was adjusted by choline hydroxide. In this case, the concentration of salts added was adjusted to 0.4 M by choline chloride. Except where otherwise noted, the motility medium was supplemented with 10 mM serine, since serine was found to induce smooth swimming in the V. alginolyticus cells for several minutes and this avoided the complication caused by the frequent changes in the swimming direction of the cells. For E. coli, the above motility medium was used although the concentration of specified salt was 0.14 M.

A small amount of a dense cell suspension was diluted 1000-fold by the motility medium, and motile cells were observed under a dark-field microscope. Swimming speed of the cells was measured at 25 °C within 1 min after dilution by the photographic method (20).

Measurement of Intracellular Na⁺ and K⁺ Concentrations—Intracellular Na⁺ and K⁺ concentrations were measured by atomic absorption spectrophotometry as described previously (21). In brief, cells (0.3 ml, about 0.09 mg of protein) were mixed with 3 ml of washing buffer consisting of 10 mM Tris-HCl (pH 7.2) and 0.4 M choline chloride, sonicated, and filtered immediately through a cellulose acetate filter (0.45 μm). After washing twice with 3 ml of the same washing buffer, filters were immersed in 3 ml of 5% trichloroacetic acid. Intracellular water space was 3.3 μl/mg of protein (14).

Measurement of the Membrane Potential—Membrane potential was measured by the use of a membrane-permeable radioactive cation, [3H]triphenylmethylphosphonium ion ([3H][TPMP⁺]) as described previously (20). In brief, cells (0.13 mg of protein/ml) were incubated with vigorous shaking at 25 °C for 5 min, and 10 μM of [3H][TPMP⁺] (0.2 mCi/mmol) was added. A sample was then filtered through a cellulose acetate filter and washed with 3 ml of 10 mM Tris-HCl (pH 7.2) containing 0.4 M NaCl. The radioactivity trapped on the filter was measured by a scintillation spectrophotometer. As a control for zero membrane potential, cells treated with 5% toluene were used. For E. coli, the concentration of desired cation for loading was 0.14 M and MgCl₂ was omitted from the medium up to 500 mM, and the swimming speed of the cells was measured within 1 min at 25 °C. The concentration of added salts was adjusted to 0.4 M by choline chloride.

TABLE 1
Effect of intracellular Na⁺ concentration on the membrane potential and swimming speed of V. alginolyticus Nap1

<table>
<thead>
<tr>
<th>pH of medium</th>
<th>[Na⁺]_o</th>
<th>[Na⁺]_i</th>
<th>?P</th>
<th>Swimming speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>7.0</td>
<td>50 mM</td>
<td>-110</td>
<td>15 μm/s</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>50 mM</td>
<td>-145</td>
<td>6 μm/s</td>
</tr>
</tbody>
</table>

in the medium up to 500 mM caused almost no improvement of motility, the swimming speed still being less than 7 μm/s. Thus, increase in intracellular Na⁺ caused a clear inhibition of the Na⁺-driven flagellar motors of V. alginolyticus, irrespective of the presence or absence of an inwardly directed Na⁺ gradient.

In order to test whether the increment of intracellular Na⁺ concentration caused a decrease in the Na⁺-motive force, which is the driving force for various Na⁺-coupled systems including the flagellar motors, it was measured in the absence of a Na⁺ gradient. As shown in Table I, the membrane potential (which corresponds to the Na⁺-motive force under the condition of no Na⁺ gradient) was around -100 mV at pH 7.0 and was not greatly altered in the Na⁺-loaded cells. When the pH of the medium of the Na⁺-loaded cells was increased to 8.5, the membrane potential increased to about -125 mV, but motility of the cells was still poor. These results indicate that the poor motility in the Na⁺-loaded cells is due not to a decrease in the driving force for the Na⁺-driven motors.

Restoration of Motility by Decreasing the Intracellular Na⁺ Concentration—It has been shown that the addition of K⁺ to Na⁺-loaded cells results in a decrease in the intracellular Na⁺ concentration and a compensatory increase in the intercellular K⁺ concentration (15). As shown in Fig. 2, the K⁺-induced decrease in the intracellular Na⁺ concentration is accompanied by a restoration of motility to the Na⁺-loaded cells. These results indicate that the high concentration of intracellular Na⁺ and the loading treatment did not cause any

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irreversible damage to the motility system.

With increasing added K+ concentration, the rate of Na+ efflux increased (Fig. 2A), and this increase paralleled the increase in the rate of restoration of motility (Fig. 2B). Similar results were obtained by altering the rate of Na+ efflux by varying the concentration of Na+ in the medium with a fixed K+ concentration (Fig. 3). These results indicate that the absolute concentration of intracellular Na+ itself is a determinant of the rotation rate of the Na+-driven flagellar motor.

**Effect of Intracellular Na+ Concentration on Na+-coupled Transport Systems**—Tokuda et al. (15) reported that the Na+-coupled AIB transport system in wild-type cells of *V. alginolyticus* was strongly inhibited in Na+-loaded cells and that the addition of K+ restored the transport activity. As shown in Fig. 4A, we confirmed that the AIB transport in Na+-loaded Nap1 cells was potently inhibited even in the presence of 400 mM Na+ in the medium, and the addition of K+ to the medium restored almost completely the transport activity. The rate of restoration of AIB transport was increased with increasing K+ concentration added to the Na+-loaded Nap1 cells (data not shown), as has been reported in the case of wild-type cells (15). Furthermore, the control cells, which contained about 50 mM Na+, showed considerable AIB transport at 50 mM or lower concentrations of Na+ in the medium, indicating that the presence of an inwardly directed Na+ gradient is not a prerequisite for the transport of AIB. Thus, the AIB transport system, which is considered to be much simpler than the motor system, is also affected by the absolute concentration of Na+ in the cell.

In contrast to AIB transport, another Na+-coupled substrate transport, sucrose transport (22), was only partly inhibited in Na+-loaded cells (Fig. 4B). This further indicates that the inhibition of motility and AIB transport in Na+-loaded cells does not result from a reduction of the Na+ motive force in the cell. Again, addition of K+ restored the transport activity. Thus, we conclude that the high intracellular Na+ concentration inhibits Na+-coupled systems, not by general damage to cellular physiology, but by a system-specific effect.

**Motility of K+-, Cs+-, or Choline-loaded Cells**—In contrast to Na+-loaded cells, K+-, Cs+-, or choline-loaded cells showed comparable swimming speed to the control cells (Fig. 5), although the loading treatment in the absence of Na+ caused...
some decrease in the motile fraction. These results seem to indicate that intracellular K⁺, which is the major cation in normal cells, is not essential for the rotation of the Na⁺- driven flagellar motors. K⁺ - or Cs⁺-loaded cells showed a Na⁺ dependence of the swimming speed quite similar to unloaded control cells.

Motility of Na⁺-loaded E. coli Cells — E. coli cells, which are known to have H⁺-driven flagellar motors (1), were loaded with Na⁺ by a similar loading procedure, but with a lower Na⁺ concentration (about 150 mM). In contrast to the motility of Na⁺-loaded V. alginolyticus, that of Na⁺-loaded E. coli was not affected by the intracellular Na⁺; the swimming speed of control cells (which contained less than 5 mM Na⁺) was about 16 µm/s, whereas that of Na⁺-loaded (which contained about 150 mM Na⁺) was about 13 µm/s. Thus, the H⁺-driven flagellar motors are essentially unaffected by the high Na⁺ content.

DISCUSSION

We have shown that an increase in the intracellular Na⁺ concentration causes a potent inhibition of rotation of the Na⁺-driven flagellar motors of V. alginolyticus, without affecting the energy level for motor rotation. The increase also strongly inhibited Na⁺-coupled AIB transport, but Na⁺-coupled sucrose transport much less effectively, indicating that the high intracellular Na⁺ concentration does not cause a general damage to the cellular physiology but rather modulates the activity of the Na⁺-coupled systems in a system-specific fashion. Furthermore, all the reduced activities were completely restored by decreasing the intracellular Na⁺ concentration, and the restoration rate was increased by increasing the rate of Na⁺ removal from the cell. Thus, it is reasonable to conclude that the absolute concentration of Na⁺ in the cell is a determinant of the rotation rate of the Na⁺-driven flagellar motors of V. alginolyticus.

In the case of the Na⁺-coupled substrate transport systems, various evidence supports the co-transport cycle model (23, 24), in which the transport carriers at first bind Na⁺ to the external side, then the Na⁺-bound carriers symport substrates by using the Na⁺-motive force as the energy, and finally the carriers release substrates and Na⁺ at the cytoplasmic side. Thus, the transport carriers, which are known to be transmembrane proteins (23, 24), are required to have two Na⁺-binding sites, one on the external side and the other at the cytoplasmic side. According to this model, the intracellular Na⁺ kinetically interferes with the cycle at the step of releasing Na⁺ from the cytoplasmic Na⁺-binding site, and the degree of interference is affected by the affinity of the Na⁺-binding site for Na⁺. Lutcher the affinity, the stronger the inhibition. If this is so, our results on AIB and sucrose transport in Na⁺-loaded cells of V. alginolyticus imply that the presumed cytoplasmic Na⁺-binding site of the AIB transport carrier has a higher affinity for Na⁺ than that of the sucrose transport carrier.

In the case of the Na⁺-driven flagellar motor, just as in the case of the Na⁺-coupled substrate transport systems, high intracellular Na⁺ concentrations caused a potent inhibition. This result can be rationalized mechanistically in terms of a Na⁺-binding site at the cytoplasmic side of the force-generating units in the motor. In addition, Sugiyama et al. (12) suggested the presence of a Na⁺-binding site at the external side of the force-generating unit of the Na⁺-driven flagellar motors of alkalophilic Bacillus, based on the finding that amiloride inhibits motor rotation by competing with Na⁺ in the medium. The Na⁺-driven flagellar motors of V. alginolyticus are also inhibited by amiloride in a competitive manner with Na⁺ in the medium. Thus, we can apply the cycle model to the rotation mechanism of Na⁺-driven flagellar motors as shown in Fig. 6; the force generating unit of motor binds Na⁺ at the external side (M₁) transfers Na⁺ to the cytoplasmic side (M₂), and finally releases Na⁺ to the cytoplasm. The force for motor rotation may be generated by using the Na⁺-motive force during the step of Na⁺ transfer. In this model, when the affinity of Na⁺ for M₁ (i.e., the binding constant K₁) is not too low, the increase in the intracellular Na⁺ concentration kinetically interferes with the release of Na⁺ from M₁, resulting in jamming of the cycle and so inhibiting motor rotation.

The presence of Na⁺-binding sites in the force-generating units of the motor brings some complexities into the energetics of the motor rotation. In particular, when the affinities of Na⁺ to the external and internal sides are fairly high, the absolute Na⁺ concentrations both in the medium and in the cytoplasm become important for motor rotation. This indicates that under this condition, the Na⁺ gradient has only an indirect role in motor rotation. As shown in Fig. 5, the Na⁺ dependence of the swimming speed of the K⁺-, Cs⁺-, or choline-loaded cells were quite similar to that of the unloaded control cells, although the former cells were expected to have a Na⁺ gradient larger than the control cells at any concentration of Na⁺ in the medium. These data seem to indicate that the Na⁺ gradient under these conditions has little role as the
energy source in motor rotation. As in the case of the data shown in Fig. 1, the swimming speed of alkalophilic *Bacillus* seemed to have the upper limit against the increase in the *Na*\(^+\) gradient (6, 20). These results may indicate the presence of the *Na*\(^+\)-binding site with a fairly high affinity of *Na*\(^+\) at the external side of the motors. In any case, it is not a prerequisite that the membrane potential and the *Na*\(^+\) gradient equally support motor rotation.

As shown in Figs. 2 and 3, a few-fold increase in the intracellular *Na*\(^+\) concentration from about 50 mM caused a significant inhibition of motility. This may indicate that the inhibition results from a complex kinetic event such as some cooperative interaction between the *Na*\(^+\)-binding site and the intracellular *Na*\(^+\) on motor rotation. Recent findings, in which the number of force-generating units per each motor is estimated to be about 10 (25-27), may have some relation to this phenomenon.

The cycle model has also been applied to explain the kinetic data of the *H*\(^+\)-driven flagellar motors with an idea of either tight coupling (28, 29) or loose coupling (30) between *H*\(^+\) flux and motor rotation. Our data on the *Na*\(^+\)-driven motors obtained by increasing the intracellular *Na*\(^+\) concentration provide a strong support on the cycle model, although the data do not discriminate the ideas of tight or loose coupling. Thus, it is quite likely that all the bacterial flagellar motors have essentially the same energy coupling mechanism in spite of the differences in the coupling ion. For the *H*\(^+\)-driven flagellar motors, experiments similar to ours may be possible. Actually, at the final stage of the preparation of this paper, we learned that Khan et al. (31) examined the effect of intracellular *H*\(^+\) concentration on the motor rotation in *Streptococcus*. Their results were well explained by the cycle model with a tight-coupling idea. However, in this case, we need to pay attention that the alterations in the *H*\(^+\) concentration are always associated with the changes in pH so that the results may contain, besides the energetic effect, some secondary effects of the pH changes on the motor system.

Consistently with the results of Kakinuma and Unemoto (22), we observed that the sucrose transport in *V. alginolyticus* 138-2 was tightly *Na*\(^+\) dependent. In contrast, Scholle et al. (39) reported that a sucrose transport system in a strain of *V. alginolyticus* was not *Na*\(^+\) dependent. We do not know the reason for the difference.

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