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MINI-REVIEW

Na^+-Driven Bacterial Flagellar Motors

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Abstract

Bacterial flagellar motors are the reversible rotary engine which propels the cell by rotating a helical flagellar filament as a screw propeller. The motors are embedded in the cytoplasmic membrane, and the energy for rotation is supplied by the electrochemical potential of specific ions across the membrane. Thus, the analysis of motor rotation at the molecular level is linked to an understanding of how the living system converts chemical energy into mechanical work. Based on the coupling ions, the motors are divided into two types; one is the H^+-driven type found in neutrophiles such as *Bacillus subtilis* and *Escherichia coli* and the other is the Na^+-driven type found in alkalophilic *Bacillus* and marine *Vibrio*. In this review, we summarize the current status of research on the rotation mechanism of the Na^+-driven flagellar motors, which introduces several new aspects in the analysis.

Key Words: Bacterial flagellar motor; Na^+-driven motor; alkalophilic *Bacillus*; marine *Vibrio*; amiloride; sodium motive force.

Introduction

Flagellated bacteria swim in the medium by rotating their helical flagella as propellers. Each flagellum is driven by a reversible rotary motor embedded in the cytoplasmic membrane (Silverman and Simon, 1974; Macnab, 1987). The motor is only about 20 nm in diameter, composed of more than 10 protein components, and supported rigidly by a peptidoglycan layer. The power for motor rotation is considered to be generated by some of the components located in the membrane. Therefore, flagellar motors are the molecular engines which provide an interesting subject for understanding the mechanism of mechanochemical coupling on the membrane.

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In 1977, we and Berg's group independently showed that the flagellar motors of *Bacillus subtilis* and *Streptococcus sp.* are directly powered by the electrochemical potential gradient of H^+ through the membrane, namely the protonmotive force (Matsuura *et al.*, 1977; Manson *et al.*, 1977). Later, several laboratories showed that the flagellar motors of other bacteria including *Escherichia coli* and *Salmonella typhimurium* are also driven by the protonmotive force (Glagolev and Skulachev, 1978; Macnab, 1987). By using a cell envelope system of *S. typhimurium* and *E. coli*, Eisenbach and Adler (1981) showed that none of the cytoplasmic materials was essential for motor rotation. This indicates that the influx of H^+ through the motor apparatus is the only requirement for the mechanochemical coupling of the motors. Recently, from measurements of the H^+ influx through the motor, Lowe *et al.* (1987) estimated that about 1000 protons were utilized for each rotation of the motor. Thus, it is concluded that the flagellar motors of the bacteria living in the moderate environment are the H^+-driven type. About -100 mV of the protonmotive force was required to give the full rotation speed of the motors (Khan and Macnab, 1980; Shioi *et al.*, 1980), and the membrane potential and the H^+ gradient, both of which are the components of the protonmotive force, are apparently equivalent as energy for the motor rotation (Matsuura *et al.*, 1979; Manson *et al.*, 1980).

Besides these neutrophiles, there is a group of bacteria living only in alkaline environment. Among them, obligately alkalophilic *Bacillus* strains have optimal growth at pH 10–11, where their motility is also optimum (Horikoshi and Akiba, 1982). Since the intracellular pH of these bacteria is maintained near neutral, the protonmotive force under the condition is quite small (Krulwich, 1986). In 1981, we found that the presence of Na^+ in the medium was absolutely and specifically required for motility of some strains of alkalophilic *Bacillus* (Hirota *et al.*, 1981). Further studies indicates that the motors of these bacteria are driven by the electrochemical potential gradient of Na^+, namely the sodium motive force (Hirota and Imae, 1983; Sugiyama *et al.*, 1985; Imae *et al.*, 1986). In addition to the alkalophiles, this new type of flagellar motors is also found in a marine bacterium, *Vibrio alginolyticus* (Chernyak *et al.*, 1983; Dibrov *et al.*, 1986; Tokuda *et al.*, 1988).

In this review, we summarize the characteristics of the Na^+-driven type of bacterial flagellar motors and the current status of the analysis of their energy-coupling mechanism.

Na^+-Dependent Motility of Alkalophiles and a Marine Bacterium

Motility of neutrophiles such as *B. subtilis* and *E. coli* with the H^+-driven flagellar motors is maximum at around neutral, where the protonmotive

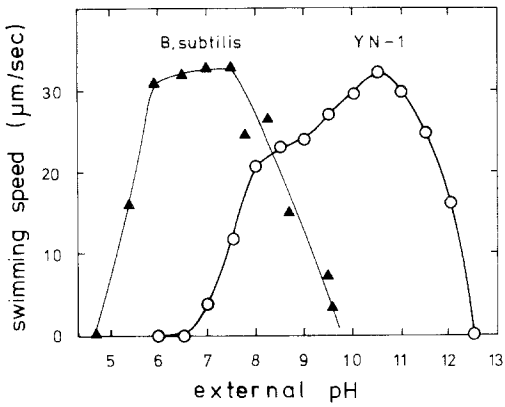

Fig. 1. Effect of medium pH on the swimming speed of *B. subtilis* cells and alkalophilic *Bacillus* YN-1 cells. Data are taken from Shioi *et al.* (1980) and Hirota and Imae (1983).

force in the cells is also maximum (Khan and Macnab, 1980; Shioi *et al.*, 1980). As shown in Fig. 1, compared with *B. subtilis*, obligately alkalophilic *Bacillus* strains show maximum motility at very alkaline pH, and significant motility is observed even at pH 12 (Hirota *et al.*, 1981; Sugiyama *et al.*, 1985). It is noteworthy that alkalophilic *Bacillus* show a swimming speed comparable to *B. subtilis* (Shioi *et al.*, 1980; Imae *et al.*, 1986). Under alkaline conditions, however, the size of the protonmotive force in alkalophilic *Bacillus* becomes smaller as the medium pH increases since the intracellular pH of these bacteria is maintained at pH 8–9 by the mechanism of pH homeostasis (Booth, 1985; Krulwich, 1986). Thus alkalophiles are vigorously motile in an environment of low protonmotive force, and this suggests that the energy source for the flagellar motors of alkalophiles is not the protonmotive force.

During the search for the energy source for motility of alkalophiles, we found that the presence of Na^+ was essential for their motility (Hirota *et al.*, 1981). As shown in Table I, only Na^+ is effective in supporting motility of alkalophilic *Bacillus*. Motility observed in the presence of Na^+ is not affected by the further addition of K^+ but slightly decreased by Li^+. Various strains of alkalophilic *Bacillus*, which were isolated from different sources, show Na^+-dependent motility (Imae *et al.*, 1986; Kitada *et al.*, 1982). In contrast, motility of the cells with the H^+-driven flagellar motors is not affected by the presence or absence of any of these cations (Manson *et al.*, 1977; Matsuura *et al.*, 1979).

Under alkaline conditions, the intracellular pH of alkalophilic *Bacillus* is maintained near neutral by the function of the Na^+/H^+ antiporter (Krulwich, 1983, 1986), indicating that the absence of Na^+ results in the

Table I. Ion Specificity for Motility of Alkalophilic *Bacillus* YN-1.[a]

Salts added	Swimming speed (μm/sec)
None	0
NaCl	16
$NaNO_3$	17
LiCl	0
KCl	0
NH_4Cl	0
RbCl	0
$CaCl_2$	0

[a]The swimming speed of the cells was measured in 25 mM Tris-HCl buffer–5 mM glucose (pH 9.0) supplemented with 15 mM of various salts. Data are taken from Hirota *et al.* (1981).

increase in intracellular pH up to the medium pH. It is likely, therefore, that the Na^+ dependence of motility of alkalophilic *Bacillus* might be the secondary effect of the Na^+-dependent pH homeostasis. However, this possibility is eliminated by the evidence that their motility is Na^+ dependent even at pH 7.5 (Hirota and Imae, 1983). A more direct evidence to eliminate the possibility is that Li^+, which does not support any motility of alkalophilic *Bacillus*, can almost equivalently substitute for Na^+ in the pH homeostasis (Sugiyama *et al.*, 1985). Matsukura and Imae (1987) reported the interesting phenomenon that the K^+ permeability of alkalophilic *Bacillus* is significantly increased by the omission of Na^+ from the medium. This means that in the absence of Na^+ in the medium, the membrane potential of the cells is collapsed at high K^+ concentration in the medium. However, in this case, too, Li^+ can equivalently substitute for Na^+ to reduce the K^+ permeability, indicating that the Na^+-dependent motility has no direct relation to this Na^+-regulated K^+ permeability.

Besides alkalophiles, Na^+-dependent motility is found in a marine bacterium, *V. alginolyticus* (Chernyak *et al.*, 1983; Dibrov *et al.*, 1986). In this case, too, only Na^+ supports motility. A characteristic property of the energy-generating systems in *V. alginolyticus* is the presence of the respiration-coupled Na^+-pump (Tokuda and Unemoto, 1982), and therefore, it is likely that the presence of this Na^+-pump may be the reason for the Na^+-dependent motility. However, this possibility is eliminated by the finding that the mutants completely defective in the respiration-coupled Na^+-pump have the same Na^+ dependence in motility as the wild type (Tokuda *et al.*, 1988).

In summary, we conclude that the Na^+ dependence is the inherent property of the flagellar motors of alkalophilic *Bacillus* and a marine *Vibrio*.

Energetics of the Flagellar Motors of Alkalophilic *Bacillus* and Marine *Vibrio*

Requirement of Na^+ for motility of alkalophilic *Bacillus* and marine *Vibrio* suggests that the flagellar motors of these bacteria may be driven by the sodium motive force, which is composed of the membrane potential and the Na^+ gradient. The role of the membrane potential in motility of alkalophilic *Bacillus* was analyzed by the use of a K^+-specific ionophore, valinomycin (Hirota *et al.*, 1981; Kitada *et al.*, 1982; Hirota and Imae, 1983). The addition of valinomycin in the presence of 60 mM or higher concentrations of K^+ in the medium caused an instantaneous inhibition of motility of alkalophilic *Bacillus* strains YN-1, 8-1, and RAB. Under these conditions, the membrane potential of these bacteria was clearly decreased, although the Na^+ gradient and the intracellular ATP content were not affected. These results indicate that the membrane potential, but not ATP, is a determinant of motility of alkalophilic *Bacillus*. The role of the Na^+ gradient in motility was analyzed simply by altering the Na^+ concentration in the medium, since the intracellular Na^+ concentration stayed almost constant at about 30 mM irrespective of the Na^+ concentration in the medium (Hirota and Imae, 1983). The swimming speed of alkalophilic *Bacillus* strains YN-1, 202-1, and RAB was increased with increasing Na^+ concentrations in the medium (Hirota *et al.*, 1981; Hirota and Imae, 1983; Imae *et al.*, 1986). These results indicate that the Na^+ gradient is also the determinant of motility of alkalophilic *Bacillus*. Consistently with this, a Na^+-specific ionophore, monensin, potently inhibited motility of these bacteria (Hirota *et al.*, 1981). We can therefore conclude that the sodium motive force is the energy source for motility of alkalophilic *Bacillus*, namely these bacteria have the Na^+-driven flagellar motors.

Hirota and Imae (1983) analyzed the quantitative relationship between the size of the sodium motive force and the swimming speed of alkalophilic *Bacillus* YN-1. Under a fixed value of the Na^+ gradient, a linear relationship between the membrane potential and the swimming speed was obtained. Furthermore, under a fixed value of the membrane potential, the relationship between the Na^+ gradient and the swimming speed of YN-1 was also linear up to about 50 mM Na^+ in the medium. As shown in Fig. 2, these data can be combined as a function of the sodium motive force, and the results indicate that the membrane potential and the Na^+ gradient are apparently equivalent to the driving force for the flagellar motors of alkalophilic *Bacillus*. The presence of a rather high threshold value of the sodium motive force (about -100 mV) is peculiar for motility of alkalophilic *Bacillus*. In the case of *B. subtilis*, the threshold protonmotive force for motility is only about -30 mV (Shioi *et al.*, 1980; Khan and Macnab, 1980), as shown in Fig. 2.

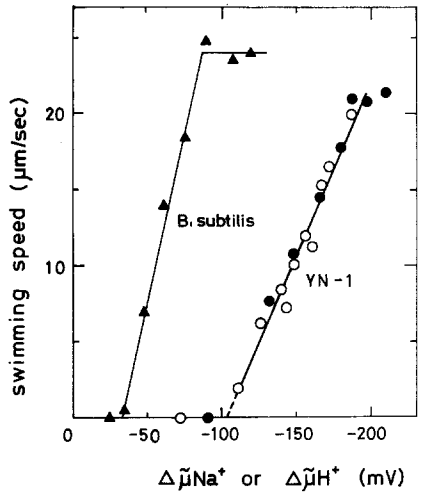

Fig. 2. Relationship between the size of the energy source and the swimming speed of *B. subtilis* cells and alkalophilic *Bacillus* YN-1 cells. The abscissa for *B. subtilis* is the protonmotive force ($\Delta\tilde{\mu}_{H^+}$) and that for YN-1 is the sodium motive force ($\Delta\tilde{\mu}_{Na^+}$). (○) Data obtained by the changes in the membrane potential; (●) data obtained by the changes in the Na^+ gradient. Data are taken from Shioi *et al.* (1980) and Hirota and Imae (1983).

Electron microscopic analysis showed that the basal body structure of the flagellar motors of alkalophilic *Bacillus* YN-1 appeared similar to that of *B. subtilis* (Hirota, N., Kamiya, R., and Imae, Y., unpublished data). This suggests that the difference in the Na^+-driven motors and the H^+-driven motors is only the difference in the coupling ion for motor rotation. It is noteworthy that, compared with neutrophiles, these alkalophilic *Bacillus* have a significantly smaller size of flagellins as a component of flagellar filaments, although the structure of the filaments is essentially the same (Guffanti and Eisenstein, 1983; Imae *et al.*, 1986).

In the case of *V. alginolyticus*, the respiration-coupled Na^+-pump generates the sodium motive force (Tokuda and Unemoto, 1982). Skulachev's group (Chernyak *et al.*, 1983; Dibrov *et al.*, 1986) reported that motility of *V. alginolyticus* is insensitive to a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone, when the Na^+-pump is active. Furthermore, Tokuda *et al.* (1988) showed that Na^+ is absolutely required for motility of this bacterium independent of the presence or absence of the Na^+-pump. These results strongly suggest that the sodium motive force, but not the protonmotive force, is the driving force for these motors. Dibrov *et al.* (1986) examined the effect of an artificially created Na^+ gradient on motility. They used cells loaded with K^+ to reduce the intracellular Na^+ content and paralyzed by 2-heptyl-4-hydroxyquinoline-*N*-oxide and arsenate. A sudden increase in the

Na^+ concentration in the medium from 0 to 400 mM induced a transient motility, indicating that the Na^+ gradient is the determinant of motility. Since the Na^+ jump from 0 to 200 mM failed to induce motility, a rather large gradient of Na^+ seems to be required to induce motility. Requirement of the membrane potential for motility was shown by the finding that motility of the Na^+-pump-deficient mutants was sensitive to carbonyl cyanide *m*-chlorophenylhydrazone (Chernyak *et al.*, 1983; Tokuda *et al.*, 1988). From these results, it is concluded that the flagellar motor of *V. alginolyticus* is the Na^+-driven type.

From the energetic point of view, the H^+-driven type of energy coupling is suitable for neutrophiles whereas the Na^+-driven type is for alkalophiles or marine bacteria. A facultatively alkalophilic *Bacillus* YN-2000 was found to grow well in a wide pH range from 7 to 10 (Koyama *et al.*, 1983). Therefore, it would be interesting if the strain has the mechanism to switch the coupling ion between H^+ and Na^+ based on the available size of the energy source. However, Sugiyama *et al.* (1986) eliminated this possibility by showing that motility and glutamine transport of YN-2000 are fixed in the Na^+-driven type irrespective of the growth pH. Furthermore, although the growth rate of this strain is almost constant between pH 7 and 10, motility and glutamine transport show maximum at around pH 9. This may indicate that YN-2000 is evolved from an obligately alkalophilic *Bacillus*.

Specific Inhibitors for the Na^+-Driven Flagellar Motors

For rotation of the Na^+-driven flagellar motors, at least one of the force-generating units of the motor is necessary to interact with Na^+ on the medium side of the membrane and translocate it through the membrane. If the Na^+-interacting site of such unit had some similarities to that of other Na^+-coupled systems, some inhibitors for the Na^+-coupled systems would be expected to inhibit the Na^+-driven flagellar motors. Based on this idea, Sugiyama *et al.* (1988) examined several inhibitors and found that amiloride, which is a potent inhibitor of Na^+ channels and a weak inhibitor of the Na^+/H^+ antiporter of various organisms (Benos, 1982; LaBelle *et al.*, 1984), rather specifically inhibited motility of alkalophilic *Bacillus*. The chemical structure of amiloride is shown in Fig. 3. The presence of the

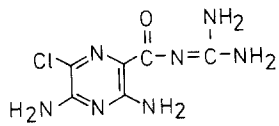

Fig. 3. Structure of amiloride.

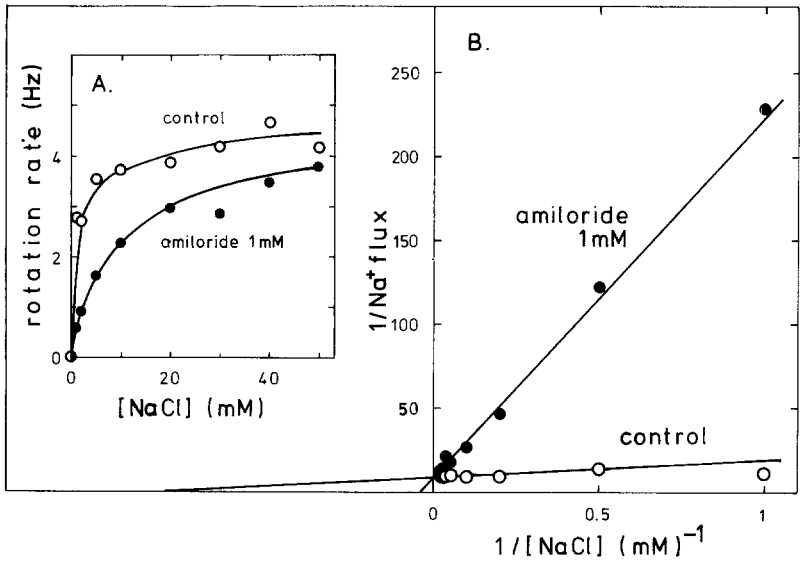

Fig. 4. Effect of amiloride and the concentration of Na^+ on the rotation rate of the flagellar motors of an alkalophilic *Bacillus*, *B. firmus* RAB. Rotation rate of the flagellar motors of RAB was measured by the method of tethered cells (Imae *et al.*, 1986). The medium for rotation measurements was 25 mM Tris-HCl buffer (pH 9.5)–5 mM glucose supplemented with various concentrations of NaCl. The supplemented salt concentration was adjusted to 50 mM by varying the concentration of choline chloride. (A) Relationship between the rotation rate and the Na^+ concentration in the presence or absence of 1 mM amiloride. (B) Kinetical analysis of the data shown in (A). The Na^+ influx through the flagellar motor was estimated by the equation described by the Sugiyama *et al.* (1988) and the unit is mV/Hz^2.

guanidinium moiety in the molecule is peculiar, as in tetradotoxin, which is a well-known potent Na^+ channel inhibitor for animals (Prince, 1988), although tetrodotoxin has no inhibitory effect on motility of alkalophilic *Bacillus* (Sugiyama, S., and Imae, Y., unpublished data).

Sugiyama *et al.* (1988) studied extensively the mode of amiloride action on motility of alkalophilic *Bacillus*. Amiloride inhibits motility of various strains of alkalophilic *Bacillus*, including YN-1, 202-1, and RAB but not of *B. subtilis*. Amiloride at the concentration required for the complete inhibition of motility of alkalophilic *Bacillus* does not affect the membrane potential, the intracellular pH homeostasis, and the ATP content of the cells. Furthermore, the activity of a Na^+-coupled amino acid transport system is only slightly affected by this concentration of amiloride. These results indicate that amiloride is a rather specific inhibitor for motility of alkalophilic *Bacillus*. An important feature of the amiloride action is the Na^+ dependence. The inhibition of motility produced by amiloride was completely restored by the increasing Na^+ concentration in the medium. The increase in K^+

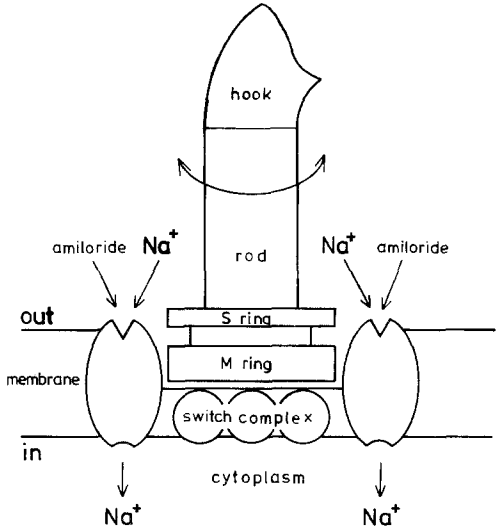

Fig. 5. A hypothetical arrangement of the Na^+-interacting unit in the structure of the Na^+-driven flagellar motors of alkalophilic *Bacillus*. Amiloride competes with Na^+ at the Na^+-interacting site located at the medium side of the unit (Sugiyama *et al.*, 1988).

concentration causes no effect but that of Li^+ rather enhances the inhibition slightly. A kinetic analysis of the data, based on the assumption that the swimming speed of the cells is a function of the rate of Na^+ influx through the flagellar motor, indicates that amiloride inhibits motility by competing with Na^+ in the medium.

Essentially the same results are obtained by analyzing the inhibitory effect of amiloride on the rotation rate of the flagellar motors of alkalophilic *Bacillus* RAB (Fig. 4A). It is clear that the inhibition is more significant at lower Na^+ concentrations. Kinetic analysis of the data also indicates that amiloride inhibits motor rotation by competitively inhibiting the Na^+ influx through the motor (Fig. 4B). Therefore, as shown in Fig. 5, it is quite reasonable to speculate that the competition between amiloride and Na^+ influx occurs at the Na^+-interacting site located in the medium side of the energy-coupling unit of the Na^+-driven flagellar motors. It is noteworthy that amiloride also inhibits the flagellar motors of *V. alginolyticus* by the competitive manner with Na^+ in the medium (Atsumi, T., and Imae, Y., unpublished data).

Besides amiloride, benzamil and phenamil also inhibited motility of alkalophilic *Bacillus* (Sugiyama *et al.*, 1988). These amiloride analogs are very potent Na^+ channel inhibitors with little or no effect on the Na^+/H^+ antiporter (Asher *et al.*, 1987). This suggests that the structure of the Na^+-interacting site of the Na^+-driven flagellar motors may have some similarity with that of Na^+ channels.

Recently, we found that another amiloride analog, iodoamiloride, irreversibly inhibited motility of alkalophilic *Bacillus* when it was activated by the UV light (Sugiyama, S., Cragoe, E. J., Jr., and Imae, Y., unpublished data). Since the high concentration of Na^+ in the medium significantly protected the motors from the irreversible inhibition by UV light, the activated iodoamiloride is suggested to bind covalently near the Na^+-interacting site of the Na^+-driven flagellar motors. Therefore, this type of approach is expected to provide useful methods for identifying the Na^+-interacting unit of the Na^+-driven flagellar motors.

Mechanism of Mechanochemical Coupling in the Na^+-Driven Flagellar Motors

For both the H^+-driven type and the Na^+-driven type flagellar motors, the mechanism of mechanochemical coupling for rotation has not yet been clarified, although several theoretical models have been proposed (Khan and Berg, 1983; Läuger, 1988; Oosawa and Hayashi, 1986). In the case of the Na^+-driven motors, however, the finding of the existence of the Na^+-interacting site in the energy coupling unit of the motors seems to bring a new interpretation in the previously reported energetic data for the motor rotation. As shown in Fig. 4, the kinetic data indicate that the relationship between Na^+ concentration in the medium and the Na^+ influx through the motor is apparently of the Michaelis–Menten type, and that the Michaelis constant for Na^+ is estimated to be about 1 mM. This means that at the region of high Na^+ concentrations in the medium, the size of the Na^+ gradient cannot simply determine the rotation rate of the motor. Namely, the linear relationship between the Na^+ gradient and the rotation rate of the motor shown in Fig. 2 could exist only in a limited range of the Na^+ concentration in the medium. The apparent saturation of the swimming speed against the increase in the Na^+ concentration in the medium was observed not only in alkalophilic *Bacillus* but also in *V. alginolyticus* (Dibrov *et al.*, 1986; Hirota and Imae, 1983; Imae *et al.*, 1986; Tokuda *et al.*, 1988). Thus, we can conclude that besides the Na^+ gradient, the absolute concentration of Na^+ in the medium itself is an important determinant for the rotation of the Na^+-driven flagellar motors.

The Michaelis–Menten type of the kinetic data shown in Fig. 4 indicates that the interaction between Na^+ and the Na^+-interacting site on the motor is sufficiently fast and not a rate-limiting step. This suggests that the rate-limiting process might be in the Na^+ transfer step during the mechanochemical coupling. The simplest model for the Na^+ movement during the mechanochemical coupling is as follows. The extracellular Na^+ binds to the

Na^+-interacting site located at the medium side of the energy-coupling unit of the motor. Amiloride inhibits this step. The bound Na^+ is then pulled inside by the membrane potential (inside negative), and this Na^+ movement is somehow coupled with rotation of the motor. At the cytoplasmic side, the Na^+ is released. The presence of the Na^+-interacting site at the cytoplasmic side of the motor is suggested by the kinetic data of motor rotation (Yoshida, S., Sugiyama, S., Tokuda, H., and Imae, Y., unpublished data).

Concluding Remarks

The discovery of the Na^+-driven flagellar motors in alkalophilic *Bacillus* and a marine *Vibrio* introduced a new concept on the approaches for understanding the mechanism of mechanochemical coupling for motor rotation. Based on the affinity of the motors to Na^+, specific inhibitors for the motors were discovered. From the kinetic analysis of the inhibitor action, the existence of the Na^+-interacting site at the medium side of the motor was revealed. The specific inhibitors are expected to be useful for the identification of the Na^+-interacting unit of the motors. These superior points of the Na^+-driven flagellar motors can well overcome the difficulty of genetic approaches in these bacteria.

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