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Characteristics of the H+-Translocating Adenosine Triphosphatase of *Vibrio parahaemolyticus*

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We have characterized H+-translocating adenosine triphosphatase (ATPase) in membrane vesicles of *Vibrio parahaemolyticus*. The ATPase required high concentrations (about 0.5 M) of Na_2SO_4 (or other salts) for its maximum activity. Magnesium ion stimulated the ATPase activity, but Ca^{2+} did not. The activity of ATPase was inhibited by tetrachlorosalicylanilide, an H+ conductor, but not by another H+ conductor, carbonylcyanide-*m*-chlorophenylhydrazone. The activity was strongly inhibited by dicyclohexylcarbodiimide or Zn^{2+}, and partially inhibited by azide, but not at all by vanadate.

Keywords proton-translocating ATPase; characteristic; salt dependency; inhibitor; *Vibrio parahaemolyticus*

The H+-translocating adenosine triphosphatase (ATPase) is widely distributed in biological membranes. In cell membranes of aerobic or facultative anaerobic bacteria, mitochondrial membranes or thylakoid membranes of chloroplast, the H+-translocating ATPase plays a central role in oxidative or photosynthetic phosphorylation.[1] In these processes, ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate by the H+-translocating ATPase is driven by an electrochemical potential of H+ established by the electron transport chains.

In bacteria, since this enzyme is essential for the oxidative phosphorylation, mutant cells lacking this enzyme can not grow on respiratory substrates such as succinate or lactate. Isolation of such mutants has been very useful to elucidate the role of this enzyme as well as for genetic studies.[2,3] The biochemical and genetic aspects of the H+-translocating ATPase have been extensively studied in *Escherichia coli*.[2-4] This enzyme consists of two portions, F_0 and F_1. The F_0 is the intrinsic membrane portion which serves as the H+ pathway and attachment site for F_1. The F_1 is the extrinsic portion and possesses ATPase activity. The ATPase activity of the F_1 is inhibited by azide but not by dicyclohexylcarbodiimide (DCCD). On the other hand, ATPase activity of F_0F_1 or of its membrane-bound form is inhibited by DCCD. The catalytic portion, F_1, is released from the membrane by washing it with a buffer containing ethylenediaminetetraacetic acid (EDTA), and it consists of α, β, γ, δ and ε subunits.[5,6]

Vibrio parahaemolyticus is a slightly halophilic marine bacterium, and is a major cause of food poisoning in Japan. Like *V. alginolyticus*,[7] this organism possesses a respiratory Na^+ pump.[8] The Na^+ circulation across the membranes plays important roles in *Vibrio*.[8,9] On the other hand, the role of H+ circulation across the membranes in *Vibrio* is not known. For example, it has not been clear whether *Vibrio* possesses an H+-coupled oxidative phosphorylation system or not. Dibrov *et al.*[10] reported that cells of *V. alginolyticus* grown on glucose under anaerobic conditions possessed Na^+-coupled oxidative phosphorylation. We observed oxidative phosphorylation coupled to H+ in *V. parahaemolyticus*.[11] We also observed a considerable oxidative phosphorylation occurring in mutants of *V. parahaemolyticus* lacking the H+-translocating ATPase.[11] Furthermore, we observed oxidative phosphorylation which was resistant to carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) in *V. parahaemolyticus*. It should be noted that oxidative phosphorylation in *E. coli* was sensitive to such H+ con-

ductors.[12] These observations suggested the presence of Na^+-coupled oxidative phosphorylation in this organism. In fact, we detected Na^+-driven adenosine triphosphate (ATP) synthesis in *V. parahaemolyticus*.[11] To elucidate the mechanism of oxidative phosphorylation in *Vibrio*, we have been characterizing the membrane-bound ATPase which would be involved in oxidative phosphorylation. We observed strong ATP-hydrolyzing activity which was sensitive to DCCD in everted membrane vesicles of *V. parahaemolyticus*. We also observed ATP-driven H+-translocation which was sensitive to DCCD in such vesicles.[8] These properties are very similar to those of the H+-translocating ATPase of *E. coli*. Thus, it seems that the ATPase activity we observed in membrane vesicles of *V. parahaemolyticus* represents the H+-translocating ATPase. Here we report the characteristics of this enzyme.

Experimental

Bacterium and Growth *V. parahaemolyticus* AQ3334 cells were grown aerobically at 37 °C in medium S[13] supplemented with 0.5% (w/v) polypeptone unless otherwise stated.

Preparation of Membrane Vesicles Cells were harvested in the late-exponential phase of growth, washed twice with buffer containing 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)–Tris (pH 7.5), 25 mM $MgSO_4$ and 0.3 M choline chloride, and suspended in the same buffer. Everted membrane vesicles were prepared by passing the cell suspension through a French press,[14] washed twice with the same buffer containing 0.5 mM phenylmethylsulfonyl fluoride, and suspended in the same buffer. Glycerol was added at 50% to the suspension and the mixture was stored at −80 °C until use. EDTA-treated membrane vesicles were prepared by washing the vesicles with a buffer containing 3 mM *N*-tris(hydroxymethyl)methylglycine (Tricine)–Tris (pH 8.0), 0.5 mM EDTA and 1 mM 2-mercaptoethanol, suspended in the same buffer as for the unwashed vesicles, and stored as described above. The washed fluid was used as EDTA-extract.

Assay of ATPase Activity The standard assay mixture (0.6 ml) consisted of 20 mM 2-(cyclohexylamino)ethanesulfonic acid (Ches)–Tris (pH 9.0), 4 mM $MgSO_4$, 0.5 M Na_2SO_4 and membrane vesicles (about 3 μg protein). EDTA-treated membrane vesicles were used only in the experiments as described in Fig. 2. Additions were made where indicated. After the preincubation at 37 °C for 3 min, ATP was added at 4 mM. The reaction mixture was incubated at 37 °C for 15 min, and the released inorganic phosphate was determined.[15] One unit of activity is defined as the release of 1 μmol inorganic phosphate per min.

Other Method Protein was determined by the method of Lowry *et al.*,[16] with bovine serum albumin as a standard.

Results

Effects of Salts and Divalent Cations Previously we reported some properties of ATP-hydrolyzing enzymes in the membrane vesicles of *V. parahaemolyticus*.[14] One of the enzymes was 5′-nucleotidase. This enzyme required relatively

high concentrations of Mg^{2+} (about 20 mM) and Cl^- (about 200 mM) for its maximum activity. Another one seemed to be the H^+-translocating ATPase, because it was inhibited by DCCD and the activity was lost from the membranes by washing the membrane vesicles with a buffer containing EDTA. However, the latter ATPase activity was low (the specific activity in the membrane vesicles was 0.1 to 0.4 unit/mg protein) under our experimental conditions. In the course of the study we noticed that the addition of Na_2SO_4 to the assay mixture increased the ATPase activity. Figure 1 shows the effect of Na_2SO_4 concentration on the ATPase activity in the membrane vesicles. When no Na_2SO_4 was added, little ATPase activity was detected. Maximum activity (2.2 units/mg of membrane protein) was observed at 0.4 to 0.6 M Na_2SO_4. Among the salts tested, we observed high ATPase activity with Na_2SO_4, K_2SO_4, and $NaOCOCH_3$ (Table I). Lower activity was observed with NaCl, $NaNO_3$, KCl, Li_2SO_4 and $(NH_4)_2SO_4$. All of these salts elicited the highest activity at 0.4 to 0.5 M concentrations (data not shown). Thus, although there are some preferences for ionic species, it seems that the major factor eliciting the high ATPase activity is the salt concentration. To avoid the involvement of the 5′-nucleotidase we added Na_2SO_4 to the standard assay mixture. The stimulation of the membrane-bound ATPase activity by Na_2SO_4 was not observed in *E. coli* membranes (data not shown). On the contrary, Na_2SO_4 (at higher concentrations than 50 mM) was inhibitory to the H^+-translocating ATPase in *E. coli*.

Figure 2 shows the effect of Mg^{2+} on the ATPase

activity. The ATPase activity was completely dependent on Mg^{2+}. The maximum activity was attained at about 4 mM Mg^{2+}. A plateau level was maintained up to 20 mM Mg^{2+}. Since the 5′-nucleotidase is very active at 10 to 20 mM Mg^{2+},[13] it seemed that this enzyme might work at such high concentrations of Mg^{2+} even in the absence of Cl^-. To test this possibility the ATPase activity was measured in EDTA-washed membrane vesicles, from which the F_1 portion of the H^+-translocating ATPase was removed. The 5′-nucleotidase is a tightly-bound membrane enzyme and is not released from the membrane or inactivated by the EDTA-washing.[14] The ATPase activity in the EDTA-washed membrane vesicles was very low even in the presence of 10 to 20 mM Mg^{2+} (Fig. 2). This result indicates that the 5′-nucleotidase is not active even in the presence of 10 to 20 mM Mg^{2+} when Cl^- is absent. Therefore, the high ATPase activity observed in the unwashed membrane vesicles at 10 to 20 mM Mg^{2+} seems to be due to the H^+-translocating ATPase. Among other divalent cations tested, Mn^{2+} and Co^{2+} were partially effective, but Ca^{2+}, Fe^{2+}, Zn^{2+} and Ni^{2+} were not (data not shown).

Effects of pH and Substrate The pH optimum for the ATPase activity was about 9.0 (data not shown). In our previous experiments we assayed the ATPase activity at pH 8.0.[13] At this pH, the activity was less than a half of the maximum:

Fig. 2. Effect of Mg^{2+} on the H^+-Translocating ATPase Activity in Membrane Vesicles

The ATPase activity in untreated membrane vesicles (●) or EDTA-treated membrane vesicles (▲) was measured at various concentrations of $MgSO_4$.
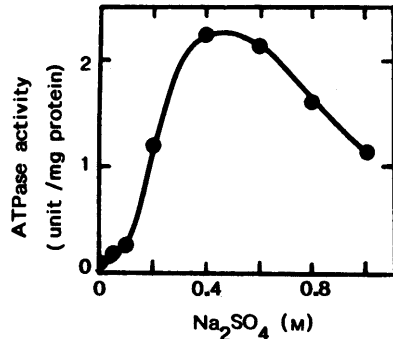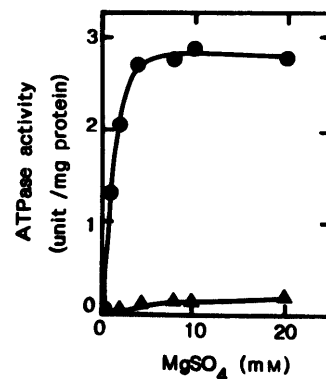

Fig. 1. Activation of the H^+-Translocating ATPase in Membrane Vesicles by Na_2SO_4

The ATPase activity in everted membrane vesicles was measured at various concentrations of Na_2SO_4.

Table I. Effects of Salts on the Membrane-Bound H^+-Translocating ATPase

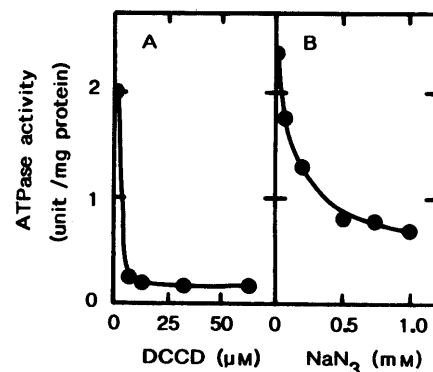| Salt[a]	ATPase activity (unit/mg protein)
—	0.25
Na_2SO_4	2.29
$NaOCOCH_3$	2.20
NaCl	1.29
$NaNO_3$	0.37
K_2SO_4	2.34
KCl	1.40
Li_2SO_4	1.17
$(NH_4)_2SO_4$	0.94

a) Each salt was added at 0.5 M.

Fig. 3. Inhibitory Effect of DCCD and NaN_3 on the H^+-Translocating ATPase Activity

The ATPase activity was measured in the presence of the indicated concentrations of DCCD (A) or NaN_3 (B). The assays in A were performed in the presence of 1% ethanol.

We also tested the effect of ATP concentration on the ATPase activity. The maximal activity was observed at 4 to 6 mM ATP (data not shown).

The substrate specificity was also tested. ATP was the best substrate, followed by guanosine triphosphate (GTP) and inosine triphosphate ITP (data not shown). Uridine triphosphate (UTP) was a poor substrate. This substrate specificity is similar to that of the F_1-ATPase in E. coli.[17] A very small amount of ADP was hydrolyzed. This hydrolysis seems to be due to the activity of the 5'-nucleotidase, which is very weak in the absence of Cl⁻.

Inhibitors One of the characteristics of the F_0F_1 type H⁺-translocating ATPase is that it is inhibited by DCCD.[18] Figure 3A shows the effect of DCCD on the ATPase activity in the membrane vesicles of V. parahaemolyticus. DCCD strongly inhibited the activity at 5 μM or above. Thus, it seems very likely that the ATPase activity we measured in the membrane vesicles in the absence of Cl⁻ was actually due to the H⁺-translocating ATPase. We observed the fluorescence quenching of quinacrine, which represents formation of a pH gradient due to the H⁺ translocation, when ATP was added to the membrane vesicles.[8] Such H⁺ translocation was inhibited by DCCD.

Azide has been reported to be a potent inhibitor of the membrane-bound ATPase or F_1-ATPase in E. coli.[17,19] Azide inhibited the membrane-bound ATPase in V. parahaemolyticus to some extent (Fig. 3B). We observed about

Fig. 4. Inhibition of the H⁺-Translocating ATPase Activity by Zn^{2+} or Ni^{2+}

The ATPase activity was measured in the presence of various concentrations of $ZnSO_4$ (●) or $NiCl_2$ (▲).

Fig. 5. Effect of TCS and CCCP on the H⁺-Translocating ATPase Activity

The ATPase activity was measured in the presence of various concentrations of TCS (●) or CCCP (▲). The assays were performed in the presence of 1% ethanol.

40% inhibition at 0.1 mM azide and 70% inhibition at 1 mM.

As has been reported previously,[13] Zn^{2+} and Ni^{2+} strongly inhibited the membrane-bound 5'-nucleotidase in V. parahaemolyticus. It seemed possible that only 5'-nucleotidase was inhibited by Zn^{2+} or Ni^{2+}. The addition of Zn^{2+} or Ni^{2+} to the assay mixture of the H⁺-translocating ATPase showed very low activity of the ATPase in the presence of these divalent cations. Thus, we tested their effect on the H⁺-translocating ATPase. Figure 4 shows the effect of concentration of Zn^{2+} or Ni^{2+} on the ATPase activity. Zinc strongly inhibited the ATPase activity and Ni^{2+} inhibited it at higher concentrations. Mn^{2+}, Ca^{2+}, Co^{2+} and Fe^{2+} (at 1 mM) did not have a significant effect on the ATPase activity when 4 mM Mg^{2+} was present (data not shown). We also tested the effect of vanadate. No inhibition was observed by up to 100 μM vanadate (data not shown).

In the course of this study we found that tetrachlorosalicylanilide (TCS), an H⁺ conductor, strongly inhibited the ATPase activity (Fig. 5). The effect of another H⁺ conductor, CCCP, on the ATPase activity was tested to see if the inhibitory effect is related to H⁺ conduction. In contrast to TCS, CCCP did not inhibit the ATPase activity. Rather, CCCP slightly stimulated the activity at low concentrations (about 5 μM). Thus, the two H⁺ conductors had different effects on the ATPase. We observed no inhibition of the H⁺-translocating ATPase in E. coli at the same concentration range of TCS. As in the case of CCCP, slight stimulation of the ATPase activity in the E. coli membrane vesicles by low concentrations of TCS was observed (data not shown).

Discussion

The H⁺-translocating ATPase in the membrane vesicles of V. parahaemolyticus was characterized. Originally we observed very low activity of the membrane-bound H⁺-translocating ATPase.[14] Under the optimal conditions described in this paper, the ATPase activity was quite high (2 to 3 units/mg of membrane protein). Thus, the activity of the H⁺-translocating ATPase in V. parahaemolyticus is comparable to that in E. coli.[20] Although most of the properties of this enzyme, such as DCCD-sensitivity, Mg^{2+} requirement, substrate specificity, pH profile, removal of the F_1 portion from the membrane by EDTA-treatment, and the presence of two major subunits, α and β,[11] are very similar to those of the E. coli enzyme, there are several remarkable characteristics of this enzyme in V. parahaemolyticus.

First, the H⁺-translocating ATPase in V. parahaemolyticus was greatly stimulated (about 10-fold) by Na_2SO_4 or other salts. The maximum stimulation was attained at about 0.5 M Na_2SO_4 or K_2SO_4. Such stimulation was not observed in the H⁺-translocating ATPase in E. coli. On the contrary, Na_2SO_4 or K_2SO_4 inhibited the enzyme in E. coli at concentrations higher than 50 mM. Since V. parahaemolyticus is a halophilic marine bacterium, this halophilicity in the H⁺-translocating ATPase might be reasonable. A similar halophilic property has been reported in the F_1-ATPase of thermophilic bacterium PS3.[21] Previously we reported the properties of the membrane-bound 5'-nucleotidase of this organism.[13] This enzyme was also stimulated by high concentrations of NaCl (about 0.2 M). Thus, halophilicity might be a common property of the membrane-
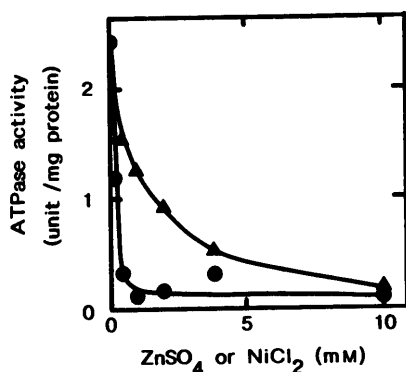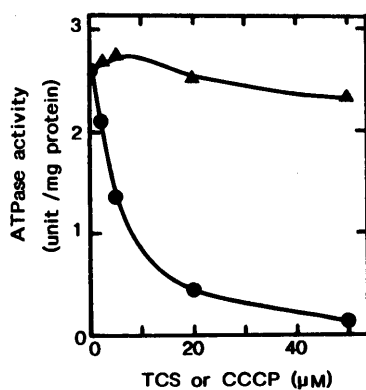
bound enzymes in marine microorganisms.

Second, TCS, an H^+ conductor, strongly inhibited the H^+-translocating ATPase activity. However, CCCP, another H^+ conductor we tested, did not inhibit the ATPase. On the contrary, we observed a slight stimulation at low concentrations (about 5 μM) of CCCP. Since an H^+ conductor dissipates the electrochemical potential of H^+ which is established by the H^+-translocating ATPase, such stimulation can be ascribed to the dissipation of the back-pressure. TCS did not inhibit the H^+-translocating ATPase in the membrane vesicles of *E. coli*. Rather TCS slightly stimulated it. Therefore, TCS might be a specific inhibitor of the H^+-translocating ATPase in *V. parahaemolyticus*. At the present time, we do not know the mechanism of the TCS-inhibition. However, the differential effect of TCS on the H^+-translocating ATPase in the two microorganisms suggests a structural difference in the enzyme. TCS may be a useful tool to analyze the structural difference between the H^+-translocating ATPase of *V. parahaemolyticus* and that of *E. coli*.

Third, Ca^{2+} was ineffective in the stimulation of the H^+-translocating ATPase. It has been reported that Ca^{2+} was as effective as Mg^{2+} in the activation of the H^+-translocating ATPase in the membrane vesicles of *E. coli*.[18,19] We observed some ATPase activity stimulated by Ca^{2+} when assayed in our old system.[14] Such ATPase activity might be due to other enzyme(s), or Ca^{2+} might be effective in the stimulation of the H^+-translocating ATPase to some extent under certain conditions.

Fourth, the optimum ratio of ATP: Mg^{2+} was not necessarily 2. It has been reported in *E. coli* that the optimum ratio of ATP: Mg^{2+} was about 2 and excess Mg^{2+} reduced the ATPase activity.[17] The ATPase activity in *V. parahaemolyticus* observed at 20 mM Mg^{2+} was the same as that observed at 4 mM Mg^{2+} when the ATP concentration was 4 mM. We tested the effect of ATP concentration on the ATPase activity, and observed the maximum activity at 4 to 6 mM ATP when Mg^{2+} was 4 mM.

Fifth, Zn^{2+} strongly inhibited the H^+-translocating ATPase in *V. parahaemolyticus*, and Ni^{2+} inhibited it to some extent. It has been reported in F_1 of *E. coli* that Zn^{2+} could partially replace Mg^{2+}.[17] Thus, Zn^{2+} is not a common inhibitor for the H^+-translocating ATPase. Zinc also inhibits the 5'-nucleotidase in *V. parahaemolyticus*.[13]

We have been trying to purify the F_1 portion of the H^+-translocating ATPase of *V. parahaemolyticus* in order to characterize it and to test whether or not F_1 is responsible for the characteristics of the H^+-translocating ATPase reported in this paper. Unfortunately, however, F_1 of *V. parahaemolyticus* released from membrane vesicles by washing them with a buffer containing EDTA is unstable (unpublished observation). We are now trying to find suitable conditions for solubilization of F_1 and F_0F_1.

Recently we found that this organism exhibits CCCP-resistant oxidative phosphorylation.[11] We observed about 50% ATP synthesis in the presence of CCCP compared with that observed in its absence. Furthermore, we observed considerable oxidative phosphorylation occurring in mutants of *V. parahaemolyticus* lacking the H^+-translocating ATPase. Thus this organism seems to possess at least two different systems for oxidative phosphorylation. One system utilizes H^+ as the coupling ion and the H^+-translocating ATPase as the ATP synthesizing machinery. The other system would utilize some other cation and other ATP synthase. As reported in a few microorganisms,[10,22,23] Na^+-coupled ATP synthesis may also take place in *V. parahaemolyticus*. If such a system does exist, we would be able to detect Na^+-translocating ATPase in the membrane of this organism. We are now trying to characterize the membrane-bound ATPase(s) in the mutants of *V. parahaemolyticus* lacking the H^+-translocating ATPase.

References
1) P. Mitchell, *Biol. Rev.*, **41**, 445 (1966).
2) M. Futai and H. Kanazawa, *Microbiol. Rev.*, **47**, 285 (1983).
3) J. E. Walker, M. Saraste, and N. J. Gay, *Biochim. Biophys. Acta*, **768**, 164 (1984).
4) E. Schneider and K. Altendorf, *Microbiol. Rev.*, **51**, 477 (1987).
5) P. D. Bragg, P. L. Davies, and C. Hou, *Arch. Biochem. Biophys.*, **159**, 664 (1973).
6) M. Futai, P. C. Sternweis, and L. A. Heppel, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2725 (1974).
7) H. Tokuda and T. Unemoto, *Biochem. Biophys. Res. Commun.*, **102**, 265 (1981).
8) T. Tsuchiya and S. Shinoda, *J. Bacteriol.*, **162**, 794 (1985).
9) H. Tokuda and T. Unemoto, *J. Biol. Chem.*, **257**, 10007 (1982).
10) P. A. Dibrov, R. A. Lazarova, V. P. Skulachev, and M. L. Verkhovskaya, *Biochim. Biophys. Acta*, **850**, 458 (1986).
11) Y. Sakai, C. Moritani, M. Tsuda, and T. Tsuchiya, *Biochim. Biophys. Acta*, **973**, 450 (1989).
12) T. Tsuchiya, *J. Biol. Chem.*, **251**, 5315 (1976).
13) Y. Sakai, K. Toda, Y. Mitani, M. Tsuda, S. Shinoda, and T. Tsuchiya, *J. Gen. Microbiol.*, **133**, 2751 (1987).
14) Y. Sakai, Y. Sumida, M. Tsuda, S. Shinoda, and T. Tsuchiya, *Chem. Pharm. Bull.*, **35**, 3771 (1987).
15) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).
16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. L. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
17) H. Kobayashi and Y. Anraku, *J. Biochem.* (Tokyo), **71**, 387 (1972).
18) M-P. Roisin and A. Kepes, *Biochim. Biophys. Acta*, **275**, 333 (1972).
19) D. J. Evans, *J. Bacteriol.*, **100**, 914 (1969).
20) T. Tsuchiya and B. P. Rosen, *J. Biol. Chem.*, **250**, 7687 (1975).
21) K. Takeda, M. Hirano, H. Kanazawa, N. Nukiwa, Y. Kagawa, and M. Futai, *J. Biochem.* (Tokyo), **91**, 695 (1982).
22) S. W. Carper and J. R. Lancaster, *FEBS Lett.*, **200**, 177 (1986).
23) W. Hilpert, B. Schink, and P. Dimroth, *EMBO J.*, **3**, 1665 (1984).