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Characterization of the membrane-bound ATPase from a facultatively anaerobic alkalophile

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We have studied the properties of membrane-bound ATPase of a facultatively anaerobic alkalophile. The enzyme could not be solubilized without detergent, suggesting an integral membrane protein. The activity was accelerated by NH_4^+ and acetate anion, and inhibited by NO_3^-. The enzyme required Mg^{2+} or Mn^{2+} as a divalent cation for the maximal activity. In addition to ATP, the enzyme utilized other triphosphates of nucleosides as a substrate, but not di- nor monophosphates. The enzyme was suggested to crossreact with an antibody against the α-subunit of Na^+/K^+-ATPase from dog kidney.

Introduction

The neutrophilic bacteria (neutrophiles), which exhibit an optimum growth in neutral region, mostly utilize a proton electrochemical potential ($\Delta\bar{\mu}_{H^+}$), a sum of transmembrane pH gradient (ΔpH) and membrane potential ($\Delta\psi$) for active transport of solutes, ATP synthesis and motility [1–5]. The alkalophilic bacteria (alkalophiles), which grow optimally in an alkaline medium, acidify the cytoplasm and exhibit ΔpH in the opposite direction to that in neutrophiles [6,7]. Thus, the aerobic alkalophiles studied so far exhibited lower magnitudes of $\Delta\bar{\mu}_{H^+}$ as compared with those of neutrophiles, although the values were still negative (around −100 mV) depending on the magnitudes of $\Delta\psi$ (negative, inside) [6–8].

We have suggested that a facultatively anaerobic alkalophile, Ep01, exhibited ΔpH in the same orientation as those of aerobic alkalophiles by the acidification of cytoplasm through the contributions of both Donnan potential and Na^+/H^+ antiporter [9]. Since the bacterium, unexpectedly, exhibited no significant magnitude of $\Delta\psi$ (negative inside), the $\Delta\bar{\mu}_{H^+}$ of the bacterium is likely to be nearly zero or even positive [9].

The aerobic alkalophiles have been suggested to contain a much higher content of cytochrome components for the electron transport system, as compared with the aerobic neutrophiles, suggesting a special energy demand for the growth in an alkaline environment [10,11]. In spite of the deficiency of cytochrome component, the growth rate of the bacterium was comparable to those of aerobic bacteria [12]. The growth of the bacterium might depend on a unique energy metabolism, which results in unusual bioenergetic properties. Since the membrane-bound ATPase, if present, is expected to play an important role in the energy metabolism, the characterization of ATPase of the bacterium was attempted in the present study. It was suggested that the bacterium possesses a unique property of ATPase as a membrane-intrinsic protein.

Materials and Methods

Cell growth and preparation of the membranes

The facultatively anaerobic alkalophile, Ep01, was grown as described previously [9]. From freshly cultured cells, the membranes were prepared according to the procedure described previously [11,12] and suspended in 20 mM Tris-HCl (pH 8)/100 mM KCl/1 mM $MgCl_2$. The solubilization of membrane proteins with detergent was carried out by the incubation of membranes in te presence of an appropriate concentration of each detergent for 15 min at 20 °C, followed by the centrifugation at 100000 × g for 30 min. The supernatant thus obtained was used as the solubilized proteins for an assay of ATPase activity.

Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential; ΔpH, transmembrane pH gradient; $\Delta\psi$, membrane potential; SDS, sodium dodecylsulfate.

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Assay of ATPase activity

ATPase activity was assayed at 30°C in 20 mM Tris-HCl (pH 8)/2 mM ATP/2 mM $MgCl_2$/200 mM NH_4Cl as a standard condition. In some experiments, 200 mM NH_4Cl was replaced by the indicated concentration of NH_4Cl or other salts, where the adjustment of ionic strength was not done. After an appropriate reaction time, the liberation of P_i from ATP was estimated as described previously [13]. 1 U of the activity was expressed as 1 μmol of ATP hydrolyzed/min.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 0.1% SDS was carried out at 10% gel concentration by the method of Laemmli [16]. Standard proteins for the estimation of molecular weight in SDS-PAGE were lysozyme (EC 3.2.1.17), lactate dehydrogenase (EC 1.1.1.27), catalase (EC 1.11.1.6), bovine serum albumin, phosphorylase *b* (EC 2.4.1.1) and myosin. The same procedure as that for SDS-PAGE was applied for polyacrylamide gel electrophoresis in the presence of MEGA-10 (decanoyl *N*-methylglucamide, Wako, Japan) except that the gel concentration was changed to 5% and 15 mM MEGA-10 was used instead of 0.1% SDS. Active staining for ATPase activity of polyacrylamide gel containing MEGA-10 was performed as described by Lübben and Schäfer [17].

Immunoblotting

For immunoblotting, proteins were transferred by electrophoresis from SDS-polyacrylamide gel to poly(vinylidene fluoride) membrane (Clear Blot Membrane-P, Atto, Japan). After transfer, the membrane was cut into strips. One of the strips was stained with Coomassie brilliant blue for the detection of protein bands. Another strip was incubated with antibodies raised in rabbits against the purified α subunit of Na^+/K^+-ATPase from dog kidney [18]. The antigen–antibody reaction was detected immunologically with anti-rabbit IgG-alkaline phosphatase conjugate (Promega, U.S.A.). The antibody, which was prepared as described by Bailey [19], was a generous gift from Dr. M. Yoshida, Tokyo Institute of Technology, Tokyo.

Protein determination

Protein concentration was determined by the methods of Gornall et al. [20] and Lowry et al. [21].

Results and Discussion

When the assay of ATPase activity was carried out at 30°C in the presence of 2 mM ATP/2 mM $MgCl_2$/200 mM NH_4Cl (pH 8), the membrane fraction isolated from freshly cultured bacteria exhibited a specific activity of 0.08 U/mg protein. The optimum pH for the activity was around 8, although approx. 90% of the activity at pH 8 was detected at pH 7 and 9. When stored at 4°C, the activity of the membrane-bound ATPase decreased to 70% of the original activity after 24 h of preparation. Since the enzyme was much less stable when stored at room temperature (almost complete loss of activity after 24 h of preparation), the inactivation of the enzyme at low temperature seems to be distinct from a cold-lability, which is a usual property of F_1-type ATPases except the enzyme from thermophiles [15,22–24]. The supernatant obtained by centrifugation after treatment of the membranes with 5 mM EDTA for 5 h at pH 8 exhibited essentially no activity. When the solubilization of the enzyme was attempted using the various concentrations of detergents such as sodium cholate, lysolecithin, $C_{12}E_8$ (octaethyleneglycol *n*-dodecyl ether), heptyl thioglucoside, Triton X-100 and MEGA-10 [25,26], the highest ratio of solubilization (approx. 50%) was attained with 25 mM MEGA-10 or 0.2% Triton X-100. The necessity of detergent for solubilization suggests that the enzyme is a membrane-intrinsic protein. By solubilization the enzyme became more labile so that the activity was reduced to approx. 40% of the original activity at 4°C after 24 h of solubilization. In the following experiments, characterization of ATPase was attempted on the membrane-bound enzyme.

When the activity of membrane-bound ATPase was measured in the presence of 200 mM NH_4Cl, NaCl and KCl, respectively, the initial rate of ATP hydrolysis was around 1.5-times higher in the presence of NH_4Cl than that in the presence of NaCl and KCl (Fig. 1). In the presence of NH_4Cl, the increase in ATP hydrolysis proceeded almost linearly for 15 min after the initiation of reaction, whereas the rate of ATP hydrolysis in the presence of NaCl or KCl began to decline around 10 min after the initiation of reaction.

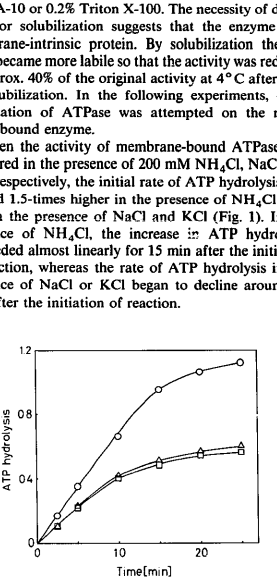

Fig. 1. ATP hydrolysis in the presence of 200 mM NH_4Cl (○), NaCl (△) and KCl (□). NH_4Cl used under standard condition for the assay of ATPase activity was then replaced by NaCl and KCl, respectively. ATP hydrolysis was expressed in μmol P_i liberated/mg protein.

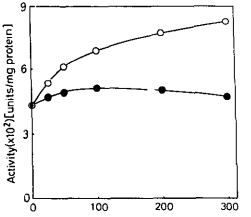

Fig. 2. Effect of concentration of NH_4Cl (○) and KCl (●) on the initial rate of ATP hydrolysis. For the assay of activity, 200 mM NH_4Cl in a standard condition was replaced by varying concentrations of NH_4Cl and NaCl, respectively.

When the assay was carried out in the presence of varying concentrations of NaCl, the initial rate of ATP hydrolysis increased slightly with increase of NaCl concentration up to 100 mM, and then decreased gradually (Fig. 2). In the presence of varying concentrations of NH_4Cl, the initial rate increased with increase in concentration even up to 300 mM. In the presence of chloride salts of other monovalent cations, K^+, Li^+, Rb^+ and Cs^+, respectively, the initial rate was almost equivalent to that in the presence of NaCl in the concentration range from 0 to 300 mM (data not shown). These results suggest that the membrane-bound ATPase is activated specifically by NH_4^+ as a monovalent cation. Cation-transport ATPases such as Na^+/K^+-ATPase, Ca^{2+}-ATPase and K^+-ATPase have been suggested to be activated specifically by the ion to be translocated [27–30]. In these cases, the activating effects of ions were detected at low concentration level, which did not exceed 20 mM. Since the optimum concentration of NH_4Cl for the enzyme activation seemed to be higher than 300 mM, it is unlikely that the enzyme functions in the translocation of NH_4^+.

In order to examine the effect of anions on the enzyme, the activity was measured in the presence of sodium or ammonium salts of various anions (Table I). The enzyme was inhibited by NO_3^- and activated by acetate ion. Inhibition by NO_3^- has been suggested in an anion-sensitive ATPase of lizard gastric mucosa [31], H^+-ATPase in fungi [31,33] and some bacterial ATPases [34,35]. Acetate and NH_4^+ seem to activate the enzyme independently in an additive manner.

When measured in the presence of 2 mM ATP, 200 mM NH_4Cl and varying concentrations of $MgCl_2$, ATPase activity increased with increase in $MgCl_2$ concentration, and exhibited a maximal and steady value in the range 2–5 mM. The optimum ratio of Mg^{2+} to ATP for the activity seems to vary depending on the type of

TABLE I

Effect of various salts on the ATPase activity

The activity was measured in the presence of 100 or 200 mM salt as indicated, which was added instead of 200 mM NH_4Cl under standard conditions.

Addition	Activity ($\times 10^2$) (U/mg protein)	
	100 mM	200 mM
NaCl	5.0	4.9
$NaNO_3$	3.0	2.6
$NaHCO_3$	3.6	4.6
$NaHSO_4$	5.9	–
Sodium acetate	6.7	7.0
NH_4Cl	6.7	7.6
NH_4NO_3	4.8	4.6
NH_4 acetate	9.1	10.4
NH_4 formate	6.4	–

enzyme. For instance, E_1E_2-type ATPases such as Na^+/K^+-ATPase, Ca^{2+}-ATPase and H^+-ATPase have been suggested to exhibit the highest activity when the molar ratio of Mg^{2+} to ATP was around 1.0 [27,36–38], whereas F_1-type ATPase exhibited the optimum ratio of 0.5 [15,39–42]. The difference of the optimum ratio might be ascribed to the difference of the physiological functions between these types of enzyme, which are ATP hydrolysis and synthesis. The optimum ratio of ATPase of the present bacterium was similar to those of E_1E_2-type ATPases, although it does not mean that the enzyme is the E_1E_2 type. Table II shows the ATPase activity in the presence of 2 mM chloride salts of various divalent cations. The enzyme required Mg^{2+} or Mn^{2+} for its maximal activity.

Table III shows the substrate-specificity of the enzyme. In addition to ATP, the enzyme hydrolyzed other triphosphates of nucleosides, although to a lesser extent. The enzyme seemed to utilize no diphosphate nor monophosphate as a substrate.

Several ATPases, such as Na^+/K^+-ATPase, Ca^{2+}-ATPase and H^+-ATPase, which exist as integral proteins in the membranes, have been found in the

TABLE II

Effect of various divalent cations on the ATPase activity

The activity was measured in the presence of 2 mM chloride salt of a divalent cation as indicated, which was added instead of $MgCl_2$ under standard conditions and expressed as the ratio compared to that in the presence of $MgCl_2$.

Addition	Activity (%)
None	33
$MgCl_2$	100
$CaCl_2$	34
$ZnCl_2$	32
$MnCl_2$	98
$CoCl_2$	62

TABLE III

Substrate-specificity of the ATPase

Activity was measured with 2 mM substrate as indicated, which was used instead of ATP under standard conditions and expressed as the ratio compared to that with ATP as a substrate.

Substrate	Activity (%)
ATP	100
GTP	61
ITP	45
UTP	30
ADP	3
GDP	0
IDP	1
AMP	0

eukaryotic cells [27,36,43–45]. In bacteria, only a few of the ATPases have been suggested as membrane-intrinsic proteins [30,46,47]. We are interested in the recent findings that there are some common features, including sequence homology of structural genes, between these eukaryotic and bacterial ATPases [18,30,48,49]. In a preliminary experiment, by immunoblotting of the membrane proteins separated by SDS-PAGE, we detected one protein of approx. 80 kDa which cross-reacted with an antiserum against the α-subunit, a major protein containing the active site of Na^+/K^+-ATPase from dog kidney (Fig. 3A). No positive band was observed by the reaction with control serum which did not contain the antibody. For partial purification, the membrane proteins were solubilized with 25 mM MEGA-10 and subjected to PAGE in the presence of 15 mM MEGA-10. After electrophoresis, the active staining for ATPase activity of the gel was carried out and the ATPase was purified by the extraction from the stained portion of the gel. Although the pattern of SDS-PAGE showed that the extract was still composed of several proteins, the 80 kDa protein was always present as a major protein and crossreacted with an antibody against the α-subunit of Na^+/K^+-ATPase from dog kidney. The number and amounts of other minor components varied from one preparation to the next (Fig. 3B). The results suggest that the 80 kDa protein is ATPase or an active component of the enzyme.

It has been suggested that E_1E_2-type ATPases functioning in cation transport are strongly inhibited by micromolar concentrations of vanadate [29,43,45,47,50,51]. When the ATPase activity of the bacterium was measured in te presence of 50 and 200 μM vanadate, the extent of inhibition was only 5 and 20%, respectively. The cation-specificity of the ATPase of the bacterium differs from those of the cation-transport ATPases studied so far [27–30,46]. The bacterium did not exhibit a significant magnitude of membrane potential [9] which could be generated by cation transport.

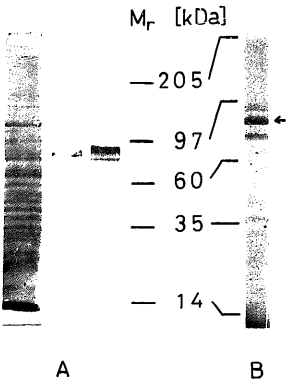

Fig. 3. Results of Western blotting (A) and SDS-PAGE (B). (A) The left lane shows the composition of membrane proteins; the middle and right lane, the positive band showing the immunological crossreaction with antibody against the α-subunit of Na^+/K^+-ATPase in the membranes and partially purified fraction, respectively. The gels for electrophoresis were loaded with 40 and 10 μg protein for membranes and purified fraction, respectively. (B) The protein bands showing the components of the partially purified fraction. The arrow shows the 80 kDa protein. The gel was loaded with 20 μg protein.

These results might suggest that the ATPase of the bacterium is not involved in the cation transport. It has been suggested that the aerobic alkalophiles require an appreciable amount of energy for growth in an alkaline medium [10,11]. To grow at alkaline pH without efficient energy production through oxidative phosphorylation, the bacterium, Ep01, would need a specific function of ATPase for the energy metabolism. The activation of the enzyme by NH_4^+ and acetate tend to suggest that the metabolic production of these substances plays an important role for the growth. To clarify the physiological function of the ATPase, further studies will be needed and are now being attempted.

References

1 Kaback, H.R. and Barnes, E.M. Jr. (1971) J. Biol. Chem. 246, 5523–5531.
2 Harold, F.M. (1972) Bacteriol. Rev. 36, 172–230.
3 Cole, J.S. and Aleem, I.H. (1973) Proc. Natl. Acad. Sci. USA 70, 3571–3575.
4 Maloney, P.C., Kashket, E.R. and Wilson, T.H. (1974) Proc. Natl. Acad. Sci. USA 71, 3896–3900.
5 Matsuura, S., Shioi, J. and Imae, Y. (1977) FEBS Lett. 82, 187–190.

6 Guffanti, A.A., Blanco, R., Benenson, R.A. and Krulwich, T.A. (1980) J. Gen. Microbiol. 119, 79–86.
7 Guffanti, A.A., Susman, P., Blanco, K. and Krulwich, T.A. (1978) J. Biol. Chem. 253, 708–715.
8 Koyama, N. and Nosoh, Y. (1985) Biochim. Biophys. Acta 812, 296–212.
9 Koyama, N., Niimura, Y. and Kozaki, M. (1988) FEMS Microbiol. Lett. 49, 123–126.
10 Lewis, R.J., Belkina, S. and Krulwich, T.A. (1980) Biochem. Biophys. Res. Commun. 95, 857–863.
11 Koyama, N., Takinishi, H. and Nosoh, Y. (1983) FEMS Microbiol. Lett. 16, 213–216.
12 Niimura, Y., Yamazaki, F., Uchimura, T., Ohara, N., Suzuki, K. and Kozaki, M. (1988) Agr. Biol. Chem. Tokyo 51, 2271–2275.
13 Ohta, K., Kiyomiya, A., Koyama, N. and Nosoh, Y. (1975) J. Gen. Microbiol. 86, 259–216.
14 Wakabayashi, K., Koyama, N. and Nosoh, Y. (1988) Arch. Biochem. Biophys. 262, 19–26.
15 Koyama, N., Koshiya, K. and Nosoh, Y. (1980) Arch. Biochem. Biophys. 199, 103–109.
16 Laemmli, U.K. (1970) Nature 227, 680–685.
17 Lübben, M. and Schäfer, G. (1987) Eur. J. Biochem. 164, 533–540.
18 Ohta, T., Nagano, K. and Yoshida, M. (1986) Proc. Natl. Acad. Sci. USA 83, 2071–2075.
19 Bailey, G.S. (1984) in Methods in Molecular Biology (Walker, J.M., ed.), Vol.1, pp. 295–300, Humana Press, Clifton, NJ.
20 Gornall, A.G., Bardwill, C.S. and David, M.M. (1949) J. Biol. Chem. 179, 751–766.
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
22 Ishida, M. and Mizushima, S. (1969) J. Biochem. 66, 133–138.
23 Schnebli, H.P. and Abrams, A. (1970) J. Biol. Chem. 245, 1115–1121.
24 Abrams, A. and Smith, J.B. (1974) in The Enzymes (Boyer, P., ed.), Vol. 10, pp. 395–429, Academic Press, New York.
25 Hildeth, J.E.K. (1982) Biochem. J. 207, 363–366.
26 Hanzani, M., Nishifuji, K., Futai, M. and Tsuchiya, T. (1984) Biochem. J. 95, 1349–1353.
27 Skou, J.C. (1960) Biochim. Biophys. Acta 42, 6–23.
28 Priestland, R.N. and Whittam, R. (1968) Biochem. J. 109, 369–374.
29 Niggli, V., Adunyah, E.S., Penniston, J.T. and Carafoli, E. (1981) J. Biol. Chem. 256, 395–401.
30 Epstein, W., Whitelaw, V. and Hesse, J. (1978) J. Biol. Chem. 253, 6666–6668.
31 De Pont, J.J.H.H.M., Hansen, T. and Bonting, S.L. (1972) Biochim. Biophys. Acta 274, 189–200.
32 Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) J. Biol. Chem. 260, 1090–1095.
33 Bowman, E.J. (1983) J. Biol. Chem. 258, 15238–15244.
34 Nanba, T. and Mukohata, Y. (1987) Biochem. J. 102, 591–598.
35 Konishi, J., Wakagi, T., Oshima, T. and Yoshida, M. (1987) Biochem. J. 102, 1379–1387.
36 Dunham, E.T. and Glynn, I.M. (1961) J. Physiol. 156, 274–293.
37 Wheeler, K.P. and Whittam, R. (1962) Biochem. J. 85, 495–507.
38 Bowman, B.J. (1983) J. Biol. Chem. 258, 13002–13007.
39 Hachimori, A., Muramatsu, N. and Nosoh, Y. (1970) Biochim. Biophys. Acta 206, 426–437.
40 Davies, P.L. and Bragg, P.D. (1972) Biochim. Biophys. Acta 206, 273–284.
41 Kobayashi, H. and Anraku, Y. (1972) Biochem. J. 71, 387–399.
42 Rosenthal, S.L. and Matheson, A. (1973) Biochim. Biophys. Acta 318, 252–261.
43 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263–3270.
44 Bowman, B.J. and Slayman, C.W. (1977) J. Biol. Chem. 252, 3357–3363.
45 Wieczorek, L. and Altendorf, K. (1979) FEBS Lett. 98, 233–236.
46 Heefner, D.L. and Harold, F.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2798–2802.
47 Hugentobler, G., Heiz, I. and Solioz, M. (1983) J. Biol. Chem. 258, 7611–7617.
48 Fürst, P. and Solioz, M. (1985) J. Biol. Chem. 260, 50–52.
49 Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28.
50 Goffeau, A. and Slayman, C.W. (1981) Biochim. Biophys. Acta 639, 197–223.
51 O'Neal, S.G., Rhoads, D.B. and Racker, E. (1979) Biochem. Biophys. Res. Commun. 89, 845–850.