Characterization of a P-type Na\(^+\)-ATPase of a Facultatively Anaerobic Alkaliphile, *Exiguobacterium aurantiacum*\(^*\)

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A facultatively anaerobic alkaliphile, *Exiguobacterium aurantiacum*, possesses a P-type Na\(^+\)-stimulated ATPase in the membrane (Koyama, N. (1999) Curr. Microbiol. 39, 27-30). In this study, we attempted to purify and characterize the enzyme. The ATPase appears to consist of a single polypeptide with an apparent molecular mass of 100 kDa. The enzyme exhibited an optimum pH for activity at around 9. The enzyme was strongly inhibited by vanadate (50% inhibition observed at 3 μM) and forms an acylphosphate intermediate, suggesting a P-type ATPase. The enzyme, when reconstituted into soybean phospholipid vesicles, exhibited ATP-dependent \(^{22}\)Na\(^+\) uptake, which was completely inhibited by gramicidin. The reconstituted vesicles exhibited a generation of membrane potential (positive, inside). The enzyme is likely to be involved in an electrogenic transport of Na\(^+\).

Neutralophilic bacteria (neutralophiles), which exhibit an optimum growth in neutral region, mostly utilize a proton electrochemical potential (\(\Delta \mu _{H^+}\)), which is the sum of transmembrane pH gradient (\(\Delta p\)H) (alkaline, inside) and membrane potential (\(\Delta \phi\)) (negative, inside), for active transport of solutes, ATP synthesis, and motility (1-4). Alkalophilic bacteria (alkalophiles), which grow optimally in an alkaline medium, acidify the cytoplasm by Na\(^+/\)H\(^+\) antiporter system and consequently exhibit \(\Delta \mu _{H^+}\) in the opposite direction to that in neutralophiles (5-7). Thus, alkalophiles utilize a sodium electrochemical potential (\(\Delta \mu _{Na^+}\)), which is the sum of transmembrane sodium gradient (\(\Delta \mu _{Na^+}\)) (outside > inside) and \(\Delta \phi\), as the driving force for active transport and motility (8-13). Although most alkalophiles might produce \(\Delta \mu _{Na^+}\) by Na\(^+/\)H\(^+\) antiporter, which is a secondary transport system of Na\(^+\), F-type, and V-type ATPases as primary sodium pumps have been suggested in several bacteria (14-19).

It has been suggested that a facultatively anaerobic alkaliphile BL77/1, a strain of *Exiguobacterium aurantiacum*, possesses a Na\(^+\)-stimulated P-type ATPase in the membrane, which is expected to function as a sodium pump (20). In this study, purification and some characterization of the ATPase were attempted. The enzyme appears to consist of a single polypeptide with an apparent molecular mass of 100 kDa. The reconstituted vesicles of the enzyme exhibited a significant uptake of \(^{22}\)Na\(^+\). The enzyme is likely to be a P-type Na\(^+\)-transport ATPase, which might be first identified in procaryotes (17).

**EXPERIMENTAL PROCEDURES**

**Culture and Preparation of Membranes—** *E. aurantiacum* BL77/1 was grown aerobically at 37 °C as described previously except that 0.5 mM NaCl was added to the medium (20). The cells were collected at a late logarithmic phase, and the membranes were prepared according to the procedure described previously (6, 21).

**Purification of the ATPase—** The freshly prepared membranes (about 60 mg of protein) were treated with 10 mM EDTA to remove loosely bound membrane proteins. After treatment, the membranes were suspended in 20 ml of a buffer containing 20 mM Tris-HCl (pH 8), 200 mM KCl, and 1 mM MgCl\(_2\), and then 2.9 ml of 20 mM deoxy-BIGCHAP was added under magnetic stirring at 4 °C. To the supernatant obtained by the centrifugation for 20 min at 90,000 × g, ammonium sulfate was added to 85% saturation. The floating precipitate was collected by centrifugation and then resolved in 0.5 ml of H\(_2\)O. The sample thus obtained was applied on a Sepharose S-300 column (2 × 20 cm), which was equilibrated with a buffer (pH 8) containing 20 mM Tris, 20% ammonium sulfate, 1 mM MgCl\(_2\), 1 mM deoxy-BIGCHAP, and soybean lecithin (0.5 mg/ml), and then fractions of 1 ml were collected. Active fractions (14-17) were combined, and 500 mM potassium phosphate (pH 7.5) was added to give a final concentration of 25 mM. The solution was applied to a column (2 × 6 cm) of hydroxyapatite (Gigapite Biochemicals, Tokyo, Japan), which was equilibrated with a buffer containing 25 mM potassium phosphate (pH 7.5) and 1 mM deoxy-BIGCHAP. The column was washed with 5 ml of the same buffer, and the enzyme was eluted with a 30-ml linear gradient of 25 mM potassium phosphate (pH 7.5) and 700 mM potassium phosphate (pH 8.5). About 30 fractions of 1 ml were collected, and to each of them, 0.5 mg of soybean lecithin was immediately added. The most active fractions (24-28) were combined and dialyzed against 20 mM Tris-HCl (pH 8.5), 200 mM KCl, and 1.0 mM MgCl\(_2\) (Buffer A) for the characterization of the enzyme.

**ATPase Assay—** The ATPase activity was assayed by the two different procedures. Routinely, the release of inorganic phosphate by the hydrolysis of ATP at 30 °C was measured as described previously (22). The reaction was carried out in a final volume of 1 ml of 20 mM Tris-HCl buffer (pH 8.5) containing 2 mM ATP, 3 mM MgCl\(_2\) and 200 mM NaCl unless otherwise stated. One enzyme unit was defined as 1 μmol of ATP hydrolyzed/min.

When the activity of the fractions from hydroxyapatite chromatography was measured, the coupled enzyme assay was employed. The assay was carried out by continuously monitoring the oxidation of NADH at 340 nm with a linked enzyme system as described previously (23). The reaction medium contained 20 mM Tris-HCl (pH 8.5), 200 mM NaCl, 2 mM ATP, 3 mM

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‡ The abbreviations used are: \(\Delta \mu _{H^+}\), proton electrochemical potential; \(\Delta \mu _{Na^+}\), transmembrane sodium gradient; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]propanesulfonic acid; BIGCHAP, \(N,N\)-bis[3-((2-gluconamidopropyl)]cholamide; deoxy-BIGCHAP, \(N,N\)-bis[3-((2-gluconamidopropyl)]deoxycholamide.

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carried out as described previously (20). Briefly, to 200 mM, respectively, followed by trichloroacetic acid precipitation. Further treatment was the same as for the untreated samples.

Phosphorylation Reaction—Phosphorylation was conducted as described previously (20). Briefly, the purified enzyme (about 1 μg of protein) was incubated at 0 °C in a total volume of 50 μl of 20 mM Tris-HCl (pH 8.2), 100 mM KCl, 100 mM NaCl, 1 mM MgCl₂, and 1 mM β-mercaptoethanol. The reaction was initiated by the addition of 10 μM [γ-³²P]ATP (111 TBq/mmol). After 60 s, the reaction was stopped by adding 50 μl of 10% trichloroacetic acid containing 1 mM sodium phosphate. The precipitated material was washed with water and then subjected to acidic gel (25) followed by autoradiography. Lane 1, control; lanes 2 and 3, same as lane 1 except that the phosphorylated samples were treated with 0.1 M Na₂CO₃ and 0.2 M hydroxylamine, respectively. Arrow indicates the position corresponding to that of the purified enzyme.

Protein Determination—Protein concentration was determined by the Lowry assay (27) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

For the recovery of Na⁺-stimulated ATPase activity in a soluble fraction from the membranes, it was necessary to use detergent, suggesting that the enzyme is an integral membrane protein. Several detergents, such as Triton X-100, heptyl thioglucoside, polyoxyethylene 9-lauryl ether (C₁₂E₉), decanoyl N-methylglucamide, n-nonyl-β-D-thiomaltoside, sucrose monocaprate, sodium cholate, CHAPS, BIGCHAP, and deoxy-BIG-CHAP were tested for the solubilization. Among them, deoxy-BIGCHAP and CHAPS were most effective. Because CHAPS, an ionic detergent, is inadequate to the application to ion-exchange chromatography for purification, a nonionic deoxy-BIGCHAP was used for solubilization.

Fig. 1A depicts the results of SDS-polyacrylamide gel electrophoresis at different stages of the purification procedure. A
100-kDa polypeptide appeared as a major band, although there are still some faint bands, the number of which varied from one preparation to the next. Incubation of the purified sample with [$\gamma$-32P]ATP demonstrated the existence of phosphorylated intermediate with a molecular mass of 100 kDa by acidic gel electrophoresis (Fig. 1B, lane 1). Treatment of the labeled samples with 0.1 M Na2CO3 and 0.25 M hydroxylamine, respectively, released the radioactive phosphates (lanes 2 and 3), indicating the acylphophate intermediate (28). These results suggest that the purified enzyme is a P-type ATPase that contains a single polypeptide with an apparent molecular mass of 100 kDa.

The purified enzyme utilized ATP as the best substrate among the nucleotides tested. The rates of hydrolysis of CTP, UTP, dTTP, UTP, ADP and AMP relative to ATP were 8, 5, 5, 4, 0, and 9%, respectively. The enzyme is likely to hydrolyze only triphosphates of nucleosides.

Fig. 2 depicts the effect of pH on the ATPase activity. The enzyme exhibited an optimum pH for activity at around 9.

Fig. 3 depicts the concentration effect of vanadate on the ATPase activity. When vanadate concentration in the reaction medium was increased, ATPase activity was decreased sharply in the concentration range of 0.1–10 M, and a half-maximal inhibition occurred at 3 µM, which was slightly higher than that of the membrane-bound ATPase (20).

The ATPase activity of the purified enzyme depended on NaCl concentration (Fig. 4). When measured in the presence of various concentration of NaCl, the activity was increased with increasing NaCl concentration, with near-saturation at around 100 mM.

The reconstituted vesicles of enzyme exhibited a significant uptake of $^{22}$Na$^+$ when ATP was added, which was completely inhibited by gramicidin (Fig. 5). The ATP-dependent uptake of Na$^+$ is likely to be accompanied with generation of an interior positive membrane potential, which was suggested by quenching of Oxonol V, a fluorescent dye, when NaCl was added (data not shown). Similarly to the ATPase activity, the initial rate of quenching was increased with increasing NaCl concentration (Fig. 4). The membrane potential could be dissipated by gramicidin, whereas the ATPase activity was accelerated about 3-fold by 10$^{-7}$ M gramicidin. Addition of LiCl, KCl, and RbCl caused essentially no quenching, suggesting that the quenching by NaCl is specific effect of Na$^+$. These facts may indicate an electrogenic transport of Na$^+$ by the enzyme.

The result obtained in this study revealed that E. aurantia- cum BL77/1 possesses a P-type Na$^+$/transport ATPase. The growth medium of the bacterium contained approximately 0.5 M Na$^+$. When Na$^+$ in the medium was replaced by K$^+$, essentially no growth was observed. The bacterium exhibited Na$^+$-dependent uptake of amino acids such as leucine and serine, which suggests that $\Delta\mu_{Na}^+$ is utilized as a driving force of active transport of the amino acids. The purified ATPase may contribute to the generation of $\Delta\mu_{Na}^+$/Na$^+$/ATPase, a sodium pump in the plasma membrane of animal cells, is a typical P-type ATPase. In a preliminary experiment, the purified enzyme did not cross-react with an antisemur against porcine Na$^+$/K$^+$/ATPase. The enzyme might possess no common epitope with animal sodium pump. We are now attempting to isolate the structural gene of the enzyme for further characterization.

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**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. U. S. A.* 70, 3571–3575
4. Maloney, P. C., Kashiwó, E. R., and Wilson, T. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 3896–3900
5. Mandel, K. G., Guffanti, A. A., and Krulwich, T. A. (1980) *J. Biol. Chem.* 255, 7391–7396
6. Koyama, N., and Nosoh, Y. (1985) *Biochim. Biophys. Acta* 812, 206–212
7. Koyama, N., Ishikawa, Y., and Nosoh, Y. (1986) *FEBS Microbiol. Lett.* 34, 195–198
8. Koyama, N., Kyoiymiya, A., and Nosoh, Y. (1976) *FEBS Lett.* 72, 77–78
9. Guffanti, A. A., Susman, P., Blanco, R., and Krulwich, T. A. (1978) *J. Biol. Chem.* 253, 708–713
10. Krulwich, T. A. (1986) *J. Membr. Biol.* 99, 113–125

$^2$ N. Koyama, unpublished results.
11. Sugiyama, S., Matsukura, H., Koyama, N., Nosoh, Y., and Imae, Y. (1986) Biochim. Biophys. Acta 852, 38–45
12. Wakabayashi, K., Koyama, N., and Nosoh, Y. (1988) Arch. Biochem. Biophys. 262, 19–26
13. Koyama, N. (1993) Eur. J. Biochem. 217, 435–439
14. Heise, R., Müller, V., and Gottschalk, G. (1992) Eur. J. Biochem. 206, 553–557
15. Smigiel, P., Majerník, A., Polák, P., Hapala, I., and Grekšak, M. (1995) FEBS Lett. 371, 119–122
16. Speelmans, G., Poolman, B., Abee, T., and Koning, W. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7975–7979
17. Dimroth, P. (1997) Biochim. Biophys. Acta 1318, 11–51
18. Kaieda, N., Wakagi, T., and Koyama, N. (1998) FEMS Microbiol. Lett. 167, 57–61
19. Murata, T., Takase, K., Yamatao, I., Igarashi, K., and Kakinuma, Y. (1997) J. Biol. Chem. 272, 24885–24890
20. Koyama, N. (1999) Curr. Microbiol. 39, 27–30
21. Ohita, K., Kiyomiya, A., Koyama, N., and Nosoh, Y. (1975) J. Gen. Microbiol. 86, 259–260
22. Koyama, N., Koshiya, K., and Nosoh, Y. (1980) Arch. Biochem. Biophys. 199, 103–109
23. Koyama, N. (1996) Anaerobe 2, 123–128
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Dame, J. B., and Scarborough, G. A. (1980) Biochemistry 19, 2931–2937
26. Holloway, P. W. (1973) Anal. Biochem. 53, 304–308
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
28. Hugentobler, G., Heid, I., and Solioz, M. (1983) J. Biol. Chem. 258, 7611–7617
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