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Properties of membrane bound ATPase in *Escherichia coli* K12 and its L-form NC7

Shigeru NAKANO and Tetsuo ONODA

Department of Biology, Faculty of Science, Shimane University,
Matsue 690, Japan

Membrane bound ATPase of L-form NC7 exhibited a pH optimum of ca. 9.0, and hydrolyzed selectively ATP with the Km value of 0.55 mM. ADP inhibited competitively the hydrolysis of ATP with the Ki value of 0.22 mM. Ca^{2+} and Mg^{2+} as divalent cation were required for the activation of this enzyme, and of these cations optimal molar ratio to ATP were 4:5 in calcium at pH 8.5 and about 2:5 in magnesium. In the presence of calcium, the enzyme was inhibited by increased concentration of monovalent cations, although in the presence of magnesium, the enzyme was relatively insensitive to their ions. Azide, PCMB and DCCD strongly inhibited the enzyme activity. The enzyme was labile under the temperature below 0–4°C, but addition of 20% methanol to the storage buffer prevented it from their inactivation. The enzyme was stable when it was preincubated below 60°C for 5 mim. Membrane bound ATPase of L-form NC7 was essentially similar to that of wild type.

Introduction

When bacteria, which is deficient in cell wall, were cultured in hypersalts-medium containing 2% NaCl as an osmotic stabilizer, the cells were exposured to crisis that is due to the inward diffusion of NaCl and water as a result of colloidosmotic and Donnan forces caused by intracellular nondiffusable macromolecules. Organisms, such as *Mycoplasma sp.*, are known to be able to maintain their cell volume and a constant intracellular ion concentrations in the presence of glucose by an energy dependent extrusion of NaCl and water from the cell (Linker and Wilson, 1985a and b).

L-form, deficient a cell wall, also would be under the similar environmental condition to *Mycoplasma sp.*. In the previous studies with L-form (NC7), which is defective in cell wall and derived from *E. coli* strain K-12, we indicated that when 340 mM of NaCl as a osmotic stabilizer was used, 1 mM $CaCl_2$ was required for the growth of cells, but when 340 mM KCl was used in place for NaCl, Ca^{2+} of the same concentration reversiblelly inhibited it (Onoda *et al.*, 1987). On the effect of $CaCl_2$ in KPY medium, further studies are now in progress. Under hypertonic environment on growth of the L-form, it is seemed that the cells are not only protected osmotically by excess Na^+ of high concentration in medium, but also are given some physiological inhibition by excess Na^+ penetrated into the cells, while by the extrusion systems of Na^+, bacteria generally could maintain lower intracellular Na^+ concentration.

As the extrusion system(s) of ion(s), primary and secondary pump are known: the former link ion extrusion with the hydrolysis of ATP (Heefner and Harold, 1980a; Heefner *et al.,* 1980b; Linker and Wilson, 1985c) or with respiratory chain (Tsuchiya and Shinoda, 1985; Udagawa *et al.,* 1986), and the latter dose it with an inwardly directed electrical proton gradient generated by H^+-translocating ATPase or electron transport system (Tsuchiya and Takeda, 1979; Brey *et al.,* 1980). In this study, we described and characterized membrane-bound ATPase of NC7 in crude membrane vesicles, as compared with those of strain 3301 and RNC7-1.

Materials and Methods

Organisms and growth media

E. coli K12 strain 3301, stable L-form NC7 induced and isolated from a wild strain, and revertant RNC7-1 that was derived from NC7 were utilized for this experiment. The medium used will be referred to as PY-medium which contained (per liter) 10 g of peptone, 5 g of yeast extract, 2 g of glucose, and cations (chloride salts) at pH 7.2 unless otherwise stated. NC7 was grown in PY-medium containing 340 mM NaCl and 1 mM $CaCl_2$. The cultures were ionculated with 3% of the culture grown overnight and incubated at 32°C for 18 to 20 h without aeration. The wild strain and RNC7-1 were precultured in PY-medium containing 85 mM NaCl and each cell was incubated at 32°C for 8 to 10 h against the former and for 12 to 14 h against the latter without aeration after inoculating with 1% of an overnight culture. Growth was monitored by measuring the absorbance at 600 nm on a Hitachi model 100–10 spectrophotometer.

In most incubation of NC7, routine observation of culture by phase-contrast microscopy, insured that it was free of other bacteria as contaminants, and 100 U/ml of penicillin G was added to the medium to prevent contamination.

Preparation of membrane

NC7 was grown to the late-logarithmic phase and collected by centrifugation at 8,000 g max for 10 mim, the pellet was washed three times with 30 mM Tris-HCl (pH 8.0)-0.3 M sucrose and then suspended in 5 mM Tris-HCl (pH 8.0). After addition of $MgCl_2$ (final conc. 2 mM) and DNase I (final conc. 0.01 mg/ml), the suspension was incubated at 37°C for 20 mim and then sonicated in an ice bath to form everted membranes. The suspension was centrifuged at 1000 g max for 10 mim at 4°C to remove unbroken cells. The supernatant was pelleted by centrifugation at 42,000 g max for 50 mim and its pellets were suspended in 25 mM Tris-HCl (pH 7.2)-20% methanol and dialyzed twice against the same solution as resuspended. Membrane was stored at 0–4°C for a week.

On the wild strain and RNC7-1, their cells were harvested at the late-logarithmic phase, and protoplast occurred after elimination of cell wall by the method of Osborn *et al.* (1972), was treated with the same method as described above. All steps were performed at 0–4°C if not stated otherwise.

Assay of ATPase activity

In a standard assay, reaction mixture (0.6 ml) contained 20 mM Tris-HCl (pH 8.5), 0.01 mg of bovine serum albumin, 1 mM $MgCl_2$, 2.5 mM ATP-Na_2 or ATP-Tris salts and 0.05 to 0.06 mg of membranes. In some experiments, indicated cations (chloride salts) and compouds were added, respectively. Reactions were performed at 37°C and initiated by the addition of ATP. The reaction was terminated by addition of 0.1 N HCl (0.3 ml). After centrifugation at 2,500 g max for 10 mim at room temperature, the supernatants (0.6 ml) were used for the determination of inorganic phosphate by the method of Fiske and Subbarow, with the slight modification described by Josse (1966). Assay was performed under the condition that activity was proportional to incubation time and amount of membrane added. Non-enzymatic degradation of ATP under these conditions was less than 0.4% of total phosphorus. Activity was expressed in term of protein measured by the method of Lowry with bovine serum albumin as standard, or also done as the percentage of the activity obtained with a standard reaction (100%).

Reagents

ATP-Na_2 was obtained from Wako Pure Chemical Industries, Ltd. ATP-Tris salts, sodium orthovanadate, oligomycin and carbony1 cyanide-m-chloropheny1 hydrazone were purchased from Sigma Chemical Co. Sodium azide, p-chloromercuribenzoate and N,N'-dicyclohexylcarbodiimide (DCCD) were obtained from Nakarai Chemicals, Ltd. Ouabain was obtained from Boehringer Mannheim. Other reagents used were of analytical grade.

Results and Discussion

General properties

The ATPase activity of each bacterial membrane was measured by determining the rate of release of Pi from ATP. On the sample of NC7, the pH optimum for ATP hydrolysis was approximaitely 9.0 in 20 mM glycine-NaOH buffer or 20 mM Tris-HCl buffer (Fig. 1). 20 mM Tris-HCl buffer (pH 8.5) was used throughout this experiment. The enzyme of membranes was able to hydrolyze ATP (other nucleoside triphosphate not tested), but unhydrolyzed ADP, AMP, phrophosphate, p-nitrophenylphosphate and glucose-6-phosphate (data not shown). ADP was inhibitory on the hydrolysis of ATP. The Ki value for ADP and the Km for ATP were 0.22 mM and 0.55 mM, respectively (data not shown).

Stability of enzyme

In order to prepare and store the samples of membrane without inactivation and solubilization, following experiment was made. Fig. 2 shows enzymatic stability of samples stored under the indicated conditions. This sample was labile at temperature

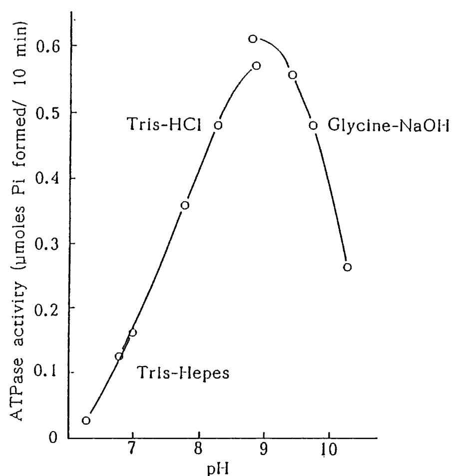

Fig. 1. Effect of pH on ATPase activity. The enzyme activity was measured under the standard conditions except for the use of the different buffer (20 mM) of pH indicated.

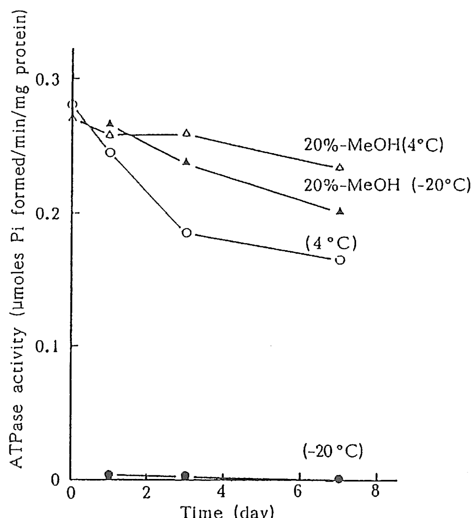

Fig. 2. Stability of ATPase. Membranes (NC7) were stored in 25 mM Tris-HCl buffer (pH 7.2), or the same buffer containing 20% methanol at the temperatures indicated. At intervals aliquots were withdawn and the activity was assayed under the standard condition.

below 0–4°C and lost 30% of initial activity when stored at 4°C for a week at a protein concentration of 4–6 mg/ml. A complete loss of the activity was confirmed when the membrane was freeze-thawed once. However, addition of methanol (20%) to the storage buffer revealed a striking protecting effect against cold inactivation (−20°C and 0–4°C) at least for a week. These results agreed with those obtained by Kobayashi *et al.* (1972).

Furthermore, heat stability of enzyme was examined. When samples of the enzyme was treated with desired temperature for 5 mim, it was stable below 60°C. The residual activities at 65°C and 70°C were about 50% and 5% of the initial activity, respectively. The treatment above 75°C completely caused inactivation (data not shown).

Obligatory requirement for divalent cations on ATPase

It was known that bacteria generally require divalent cations for activity of ATPase (Kobayashi and Anraku, 1972; Koyama *et al,* 1980; Linker and Wilson, 1985c). It was found that ATPase of L-form NC7 was also activated by the divalent cations such as Ca^{2+} and Mg^{2+}. The results obtained were shown in Table 1. There was little activity in the absence of added divalent cation. The activity by addition of 1 mM Ca^{2+}, Mn^{2+} and Co^{2+}, as chloride salts indicated that these cations were able to replace Mg^{2+} partially. However, in the presence of 1 mM Mg^{2+}, these cations showed a

Table 1. Effect of divalent cations on ATPase activity

Cation	$Mg^{2+}(+)$	$Mg^{2+}(-)$
None	100	4
$CaCl_2$	73	55
$CoCl_2$	45	26
$MnCl_2$	56	52
$CuCl_2$	44	3
$BaCl_2$	33	2

The enzyme activity was assayed under the standard conditions except that ATP-Na_2 was replaced by ATP-Tris, and that 1 mM of the cation indicated (chloride salts) was added to the reaction mixture in the presence or absence of 1 mM Mg^{2+}. The activity was expressed as the perecentage of that with 1 mM Mg^{2+} (0.257 μ moles/min per mg protein).

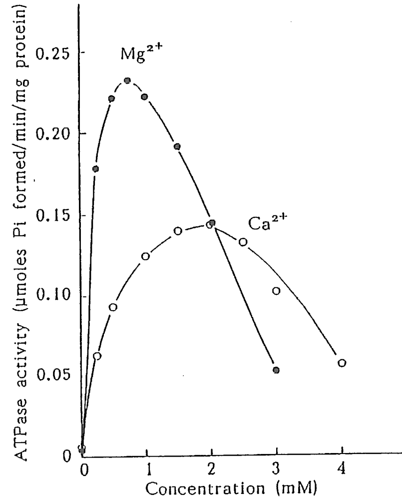

Fig. 3. Effect of Ng^{2+} and Ca^{2+} on ATPase activity. The reactions were tried under the standard conditions except that ATP-Na_2 was replaced by ATP-Tris, and that $MgCl_2$ and $CaCl_2$ at the indicated concentrations were added to the reaction micture.

competitive inhibition. Cu^{2+} and Ba^{2+} (chloride salts) could not substituted for Mg^{2+}, and they were considerably inhibitory in the presence of 1 mM Mg^{2+}. As shown in Fig. 3, in the case of 2.5 mM ATP, Ca^{2+} concentration required an optimal enzymatic activity was around 2.0 mM, and in the case of Mg^{2+}, was 0.8 mM.

Effect of monovalent cations on ATPase activity

Na^+-translocating ATPase generally revealed Na^+-stimulation of ATP hydrolysis (Shou, 1957; Hilpert *et al.*, 1984). As NC7 was grown preferably in the hyperosmolar liquid medium supported osmotically by NaCl or KCl, it is significant to examine the effects of cations on the enzyme of L-form NC7. 10 mM monovalent cations exhibited a very slight stimulation of ATPase activity (Table 2). 10 mM Na^+ plus K^+ also had no remarked effect. Furthermore, the effects of either Na^+ or K^+ at the various concentrations were examined, respectively, in the presence of 1 mM Mg^{2+} or Ca^{2+} (Fig. 4). In the presence of Mg^{2+}, 1–15 mM Na^+ caused only a little activation, while Na^+ concentrations higher than 20 mM were progressively inhibitory against the activity of ATPase; 76% at 80 mM NaCl compared with control. On the other hand, when Mg^{2+} was replaced for Ca^{2+}, the more Na^+ concentrations increased, the more the activity decreased; 25% at 80 mM NaCl. In the case of K^+, the similar results as those of Na^+ were also obtained.

These results were consistent with the report of Evans (Doyle and Evans, 1969), using membrane ghost preparation of *E. coli* K-12.

Effects of potential inhibitors on ATPase activity

Effects of inhibitors on the membrane bound ATPase were investigated (Table 3). Dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone and KCN, which were uncoupler of oxidative phosphorylation, had no effect. Ouabain, the inhibitor of the aminal cell Na^+, K^+-ATPase, failed to inhibit the enzyme. Oligomycin is known to inhibit the mitochondrial F_0F_1-ATPase, but not the bacterial F_0F_1-ATPase. Membrane bounding ATPase of NC7 was also insensitive to oligomicin. ICH_2COOH, Na_3VO_4 and Na_2HAsO_4 had no effect.

PCMB inhibited the enzyme of NC7 by 58% at 0.01 mM. Azide is known as a potent inhibitor for F_0F_1-ATPase of mitochondria and of several bacteria. Sodium azide inhibited the enzyme of NC7 by 94% at 1 mM. When 0.1 mM DCCD, which is a classical inhibitor of bacterial F_0F_1-ATPase, was added to the reaction mixture at pH 7.5, the enzyme was striking inhibited by 82%.

From the results described above, the properties of membrane bound ATPase in NC7 was consisted with that of *E. coli* reported by Kobayashi *et al.* (1972). Further, this enzyme of NC7 was similar to that of wild, although L-form NC7 grows only in hypersalts medium. It found that general characteristics of membrane bound ATPase was kept even under hypertonic circumstance.

Table 2. Effect of monovalent cations on ATPase activity

Cation	Relative activity
None	100
NaCl	106
KCl	106
LiCl	94
RbCl	102
CsCl	106
NaCl+KCl	105

The reaction condition was identical to the standard conditions except that ATP-Tris instead of ATP-Na_2, and 10 mM of monovalent cations were added. The activity obtained without monovalent cations was taken as 100 (0.311 umoles Pi formed/min per mg protein).

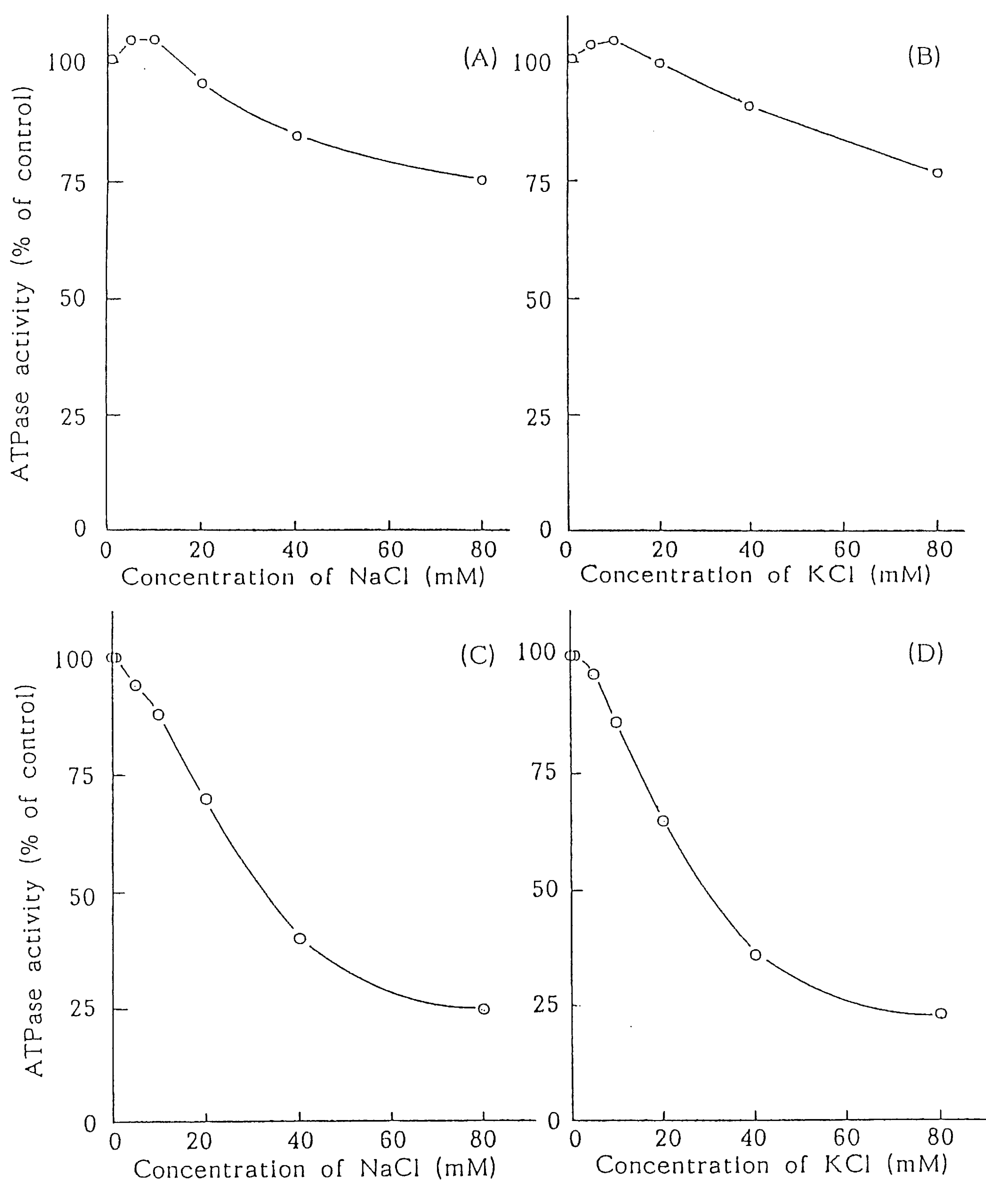

Fig. 4. Effect of monovalent cations in the presence of Mg^{2+} or Ca^{2+} on ATPase activity. (A) Effect of Na^+ in the presence of 1 mM Mg^{2+}; (B) Effect of K^+ in the presence of 1 mM Mg^{2+}; (C) Effect of Na^+ in the presence of Ca^{2+} ; (D) Effect of K^+ in the presence of Ca^{2+}. The reaction was identical to that of Table 2 except that Na^+ and K^+ at the indicated concentrations in the presence of Mg^{2+} or Ca^{2+} were added to the reaction mixture. The activity obtained without monovalent cation in the presence of each divalent cation was taken as 100. Specific activities without monovalent cation in the presence of Mg^{2+} and Ca^{2+} were 0.324 and 0.170 μmoles Pi formed/min per mg protein, respectively.

Table 3. Effect of potential inhibitors on ATPase

Compound	Concentration	Wild	RNC7-1	NC7
None		100	100	100
KCN	1.0 mM	100	104	102
CCCP	0.01 mM	106	107	102
DNP	1.0 mM	102	97	101
Ouabain	0.25 mM	100	103	101
Oligomycin	5.0 μg/ml	106	107	103
NaN_3	1.0 mM	7	9	6
DCCD	0.1 mM	77	73	86
DCCD (pH 7.5)	0.1 mM	37	26	18
PCMB	0.01 mM	44	34	42
ICH_2COOH	1.0 mM	92	97	98
Na_3VO_4	2.5 mM	103	101	97
Na_2HAsO_4	2.5 mM	96	98	91

The enzyme activity was assayed under the standard conditions except that the compound indicated was added to the reaction mixture (at pH 8.5 and pH 7.5 in the case of DCCD). The activity was expressed as a percentage of that without a compound.

¶ CCCP; Carbonyl cyanide m-chlorophenylhydrazone.
DNP; 2, 4-Dinitrophenol.
DCCD; N, N'-Dicyclohexylcarbodiimide.
PCMB; *p*-Chloromercuribenzoic acid.

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