Intracellular Na\(^+\) Regulates Transcription of the \textit{ntp} Operon Encoding a Vacuolar-type Na\(^+\)-translocating ATPase in \textit{Enterococcus hirae}*

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The Gram-positive bacterium \textit{Enterococcus hirae} has a vacuolar-type Na\(^+\)-translocating ATPase that is encoded by the \textit{ntp} operon (\textit{ntpFIKECGABDHJ}) (Takase, K., Kakinuma, S., Yamato, I., Konishi, K., Igarashi, K., and Kakinuma, Y. (1994) \textit{J. Biol. Chem.} 269, 11037–11044). Primer extension experiments identified the start site of transcription of this operon upstream of the \textit{ntpF} gene. In parallel with the increases of both Na\(^+\)-pumping activity in whole cells and Na\(^+\)-stimulated ATPase activity in the membranes, the amounts of the two major subunits (A and B) of this enzyme increased remarkably in cells grown on medium containing high concentrations of NaCl but not on medium containing KCl or sorbitol. Chloramphenicol completely abolished the increases of the enzyme activity and the amounts of A and B subunits, suggesting that the Na\(^+\)-ATPase level increased by \textit{de novo} synthesis of the enzyme with the stimulation of high concentrations of the external sodium ions. Finally, Western blot and Northern blot experiments revealed that the increase in the Na\(^+\)-ATPase level with the external Na\(^+\) was further accelerated by addition of an ionophore, such as monensin, which rendered the cell membrane permeable to Na\(^+\). These results suggest that the transcription of the Na\(^+\)-ATPase operon is regulated by the intracellular concentration of sodium ions.

All living cells show Na\(^+\) circulation across the cell membrane. This circulation is driven by active transport systems that extrude sodium ions and maintain the Na\(^+\) concentration gradient directed inward (1–3); Na\(^+\) reenters the cell via a Na\(^+\) gradient consumer, such as Na\(^+\)-coupled secondary cotransport systems, as the widespread route (4). In animal cells, the familiar Na\(^+\)-K\(^+\)-ATPase expels sodium ions, to which K\(^+\) uptake is tightly coupled. In bacteria, secondary Na\(^+\)-H\(^+\) antiporters, which are driven by the proton electrochemical gradient generated by proton pumps, are widely distributed to perform this work. However, it is now generally accepted that bacteria have evolved multiple sodium extrusion systems (primary and secondary ones) for the purpose of coping with environmental fluctuations (5).

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1 The abbreviations used are: [Na\(^+\)]\(_{in}\), the intracellular Na\(^+\) concentration; [Na\(^+\)]\(_{out}\), the external Na\(^+\) concentration; DCCD, N,N’-dicyclohexylcarbodiimide; TCS, tetrachlorosalicylanilide.
Transcriptional Regulation of E. hirae Na\(^+\)-ATPase Operon

**Table I**

| Addition | Na\(^+\)-ATPase activity | Relative amount of A and B subunits |
|----------|--------------------------|----------------------------------|
| None     | 0.02                     | 1.0                              |
| KCl      | 0.03                     | 1.2                              |
| NaCl     | 0.48                     | 17.3                             |
| Sorbitol | 0.03                     | 1.1                              |

**Materials and Methods**

**Strain and Growth Conditions**—The *E. hirae* strain used was ATCC 9790 (wild-type strain), obtained from the American Type Culture Collection. Cells were grown in the complex medium KNY (0.2% Bacto neopeptone, 0.2% Bacto yeast extract, 1% glucose, and 0.85% KH\(_2\)PO\(_4\)). The pH of the medium was adjusted to 7.5 with K\(_2\)CO\(_3\). This medium was supplemented with 2% yeast extract, 0.5% NaCl, 0.2% DCCD, and 0.5% Bacto peptone. Bacto peptone and yeast extract were used at 0.3% and 0.5% levels, respectively.

**Results**

**Sodium Specifically Increases the Amount of Na\(^+\)-ATPase**—The Na\(^+\)-ATPase from *E. hirae* is composed of two parts: a water-soluble catalytic (V\(_c\)) moiety and a membrane-embedded (V\(_m\)) moiety (26). The V\(_c\) moiety is composed of two major subunits, NtpA (65 kDa) and NtpB (52 kDa), and several minor subunits (9).

**Primer Extension**—Determination of the transcription start site of the ntp operon was achieved by the primer extension as described (24). Total RNA was extracted as described (25) from *E. hirae* cells grown in various media. The primer oligonucleotide (5\'(CTTGTGCTAAATGCGCG), which is located on the noncoding strand just downstream of the putative initiation codon (ATG) for the ntp\(_c\) gene, was end-labeled with \(\gamma\)-\(^32\)P\]ATP using polynucleotide kinase. Total RNA extract (20 \(\mu\)g) and the labeled primer (4 \(\times\) \(10^6\) cpm) were mixed and incubated at 60°C and at room temperature as described (24). The primer extension reaction was started in the reaction mixture (24) with reverse transcriptase. The product was precipitated with ethanol and analyzed on a sequencing gel with a size marker.

**Nernstian Blotting**—Total RNA was extracted as described elsewhere (25) with *E. hirae* cells grown in various media, fractionated through formaldehyde gels, and transferred to nitrocellulose membranes. A DNA fragment labeled by the random priming method (25) was used as a probe.

**Miscellaneous Methods**—The catalytic V\(_c\) moiety of Na\(^+\)-ATPase was purified as described previously (13). The cell membranes were prepared by the standard procedure as described previously (26), and, if necessary, stored frozen at \(-80\)°C. The ATPase activity was assayed in the presence of 0.2 mM DCCD by a procedure described elsewhere (21); the Na\(^+\)-stimulated ATPase activity of the membranes was determined at pH 8.5 in the presence or absence of 25 mM NaCl. Denatured polyacrylamide gel electrophoresis was carried out using the system of Laemmli with 10% polyacrylamide (27) and stained with Coomassie Brilliant Blue R-250. Protein was determined by the method of Lowry et al. (28) with bovine serum albumin as standard.

**Materials**—Enzymes for recombinant DNA techniques were purchased from TOYOBO (Tokyo, Japan) and Takara Shuzo Co. (Kyoto, Japan). \(\gamma\)-\(^32\)P\]ATP (220 TBq/mmol) and \(\gamma\)-\(^35\)S\]ATP (220 TBq/mmol) were purchased from Amersham Corp., and \(^32\)P\]NaCl was purchased from Daiichi Pure Chemical Co. (Tokyo, Japan).\(^{22}\)NaCl was purchased from ICN Biomedicals Inc. (Irvine, Calif.). All reagents used were commercial products of analytical grade.

**Results**

**Sodium Specifically Increases the Amount of Na\(^+\)-ATPase**—The Na\(^+\)-ATPase from *E. hirae* is composed of two parts: a water-soluble catalytic (V\(_c\)) moiety and a membrane-embedded (V\(_m\)) moiety (26). The V\(_c\) moiety is composed of two major subunits, NtpA (65 kDa) and NtpB (52 kDa), and several minor subunits (9). We purified the catalytic moiety, whose molecular mass is about 400 kDa, consisting of NtpA, NtpB, and NtpD (29 kDa) with a probable stoichiometry of 3:3:1 (13) (Fig. 1, lane 1); other minor subunits of V\(_c\) part were presumably dissociated during this purification procedure.

First, the immunoblotting using antiserum raised against the purified catalytic moiety was performed with the cells grown on KNY medium (limited Na\(^+\)) or KNY medium containing 0.3 mM NaCl (high Na\(^+\)) (Fig. 1). The Na\(^+\)-stimulated ATP hydrolytic activities of the membranes prepared from these cells were 0.01 (limited Na\(^+\)) and 0.25 (high Na\(^+\)) \(\mu\)mol/min/mg of protein, respectively. In parallel with an increase in the Na\(^+\)-ATPase activity in high Na\(^+\) conditions, the amounts of the two major subunits (A and B) of Na\(^+\)-ATPase increased in the membranes (Fig. 1, lanes 3 and 4). Although the purified enzyme contained the 29-kDa (D) subunit (19), this subunit was not detected clearly in this immunoblot assay (Fig. 1, lane 2); the antiserum probably contained only a small amount of antibodies against this subunit. The amounts of the A and B subunits in total cell protein also increased in high Na\(^+\) conditions (Fig. 1, lanes 5 and 6), disproving that an increase in...
Na$^+$-ATPase in membranes is caused by an elevated rate of enzyme incorporation into the membranes. We also observed a small amount of $V_1$ moiety in the soluble fraction; we do not know whether these $V_1$ moieties are the fraction released from the $V_0$ moiety in the membrane during cell disruption or into the $V_1$ pool before incorporation into the membrane (29).

By measuring the radioactivity from $^{125}$I-labeled protein A bound to the A and B subunits, the amount of Na$^+$-ATPase was compared in the cells grown in various media (Table I). Cells were grown on KNY medium containing NaCl, KCl, or sorbitol at a concentration of 1 m. The Na$^+$-stimulated ATPase activity of membranes prepared from cells grown in medium containing NaCl was 24-fold higher than the value of those from cells in KNY medium. Likewise, the amount of the A plus B subunits in total cell protein was increased 17-fold by addition of NaCl. On the other hand, both Na$^+$-ATPase activity and amounts of the A plus B subunits were little affected by addition of KCl or sorbitol (Table I). Fig. 2 shows active $^{22}$Na$^+$ extrusion from the cells grown in these media. In these experiments, DCCD, TCS, and valinomycin were all added in the assay mixture so as to block the generation of the proton gradient; sodium extrusion via the Na$^+/H^+$ antiporter was neglected. The proton gradient-independent $^{22}$Na$^+$ extrusion was observed in the cells grown at high Na$^+$ condition, but negligible in the cells in high K$^+$ or sorbitol media (Fig. 2). The effect of LiCl or CsCl on the amounts of the A and B subunits was also examined by immunoblotting. Experiments were performed at the concentration of 150 mM because these salts are toxic for this organism. Whereas the amounts of A and B subunits were increased 5.5-fold by the addition of 150 mM NaCl to the medium, the amounts increased 2.6- and 1.1-fold when cells were grown in high LiCl and high CsCl, respectively. Furthermore, the effect of other osmolytes, such as glutamate, proline, or betaine, at a concentration of 0.2 M, was negligible on the amounts of these subunits. These results indicate that an increase in the amount of Na$^+$-ATPase is the specific response to high concentration of sodium ions or lithium ions, not to the ionic strength or osmotic change.

**Sodium Stimulation of de Novo Synthesis of Na$^+$-ATPase**—Fig. 3 shows the effect of chloramphenicol on the increase in the amount of Na$^+$-ATPase. To follow the quantitative change during cell growth, the cell culture was started in KNY medium, and then 0.8 M NaCl was added. Cell growth was slowed down by addition of 0.8 M NaCl, but recovered after about a 1-h lag period (Fig. 3A, closed circles). Although the growth was slightly slower than that in medium without NaCl, the growth yields in both media were similar (data not shown). The amounts of the A plus B subunits of the Na$^+$-ATPase were limited but constant in the cells growing in this Na$^+$-depleted medium (Fig. 3B, open circles). Addition of NaCl remarkably increased the amounts of these subunits preceding the growth recovery (Figs. 3, A and B, closed circles), and at 1 h after the addition of NaCl, the amounts of these subunits increased by 7-fold of the value before addition of NaCl and 15-fold at 2 h. When chloramphenicol (100 $\mu$g/ml) was added with NaCl, an increase in the amounts of these subunits was not observed (Fig. 3B, closed triangles). Furthermore, the proton gradient-independent Na$^+$-pumping activity was not observed in the cells grown on KNY medium (Fig. 4A, closed circles); in the absence of the ionophores, however, sodium ions were actively extruded via the Na$^+/H^+$ antiporter (Fig. 4A, closed triangles) (6). By addition of 0.5 M NaCl into KTY medium, the proton gradient-independent Na$^+$ extrusion was induced (Fig. 4B). However, the addition of 100 $\mu$g/ml chloramphenicol with NaCl abolished the induction of proton gradient-independent Na$^+$-pumping activity (Fig. 4C). An increase in the Na$^+$-stimulated

**Fig. 2. Sodium-pumping activity by E. hirae cells grown in various media.** Cells were grown in KNY medium, and at $A_{600} = 0.1$, NaCl (A), KCl (B), or sorbitol (C), at a concentration of 1 M, was added to the medium. After two generations ($A_{600} = 0.4$), cells were harvested and $^{22}$Na$^+$ extrusion was assayed as described under “Materials and Methods.” DCCD (0.2 mM), TCS (5 $\mu$M), and valinomycin (5 $\mu$M) were added to the assay mixture at −5 min, and the reaction was initiated by 10 mM glucose (indicated by arrows) at 5 min. Open circles, without glucose; closed circles, with glucose.

**Fig. 3. Time course of a change of the amount of the A and B subunits of Na$^+$-ATPase during cell growth.** Cell culture was started in KNY medium, and 0.8 M NaCl and chloramphenicol (100 $\mu$g/ml) were added (arrow). At intervals, the cell growth (A) was monitored, and the amounts of the A plus B subunits (B) in total cell proteins were immunochemically measured with $^{125}$I-labeled protein A as described under “Materials and Methods.” Open circles, without additions of NaCl and chloramphenicol; closed circles, with addition of NaCl and without addition of chloramphenicol; closed triangles, with additions of NaCl and chloramphenicol.
ATPase activity of the membranes at high Na⁺ condition was also blocked by chloramphenicol (data not shown). All these results suggest that protein synthesis is required for the increase in the amount of Na⁺-ATPase. When rifamycin SV, instead of chloramphenicol, was added with NaCl, the increase in Na⁺-ATPase activity in membranes was prevented and the basal amount of Na⁺-ATPase subunits was slightly decreased (data not shown). Although we cannot exclude a possibility that a change in the rate of enzyme degradation increases the Na⁺-ATPase level at such a high Na⁺ condition, the results obtained suggest that a stimulation of de novo synthesis of the Na⁺-ATPase by sodium ions elevates the enzyme level.

In contrast to rifamycin SV, chloramphenicol did not decrease the enzyme basal level, although it inhibited the increase (Fig. 3B, closed triangles). It appears that protein synthesis was not inhibited completely by chloramphenicol under our conditions. In fact, E. hirae can grow in the presence of chloramphenicol under such conditions, although the growth rate is very low.

Sodium Regulates Transcription of the ntp Operon—Fig. 5A shows the arrangement of the ntp genes in the Na⁺-ATPase operon (12). Northern blot experiments with several DNA probes corresponding to these ntp genes have revealed that the ntp operon is composed of 11 genes: ntp-FIKKECGABDHJ. The arrow indicates the direction of the operon. The DNA segment designated by the shaded box represents the probe used for Northern blotting as shown in Fig. 7. B, primer extension. Total RNA was extracted from the cells grown in NaTY medium containing 0.5 M NaCl at pH 10.0 (lane 1), NaTY medium containing 0.5 M NaCl (lane 2) or KTY medium (lane 3). Primer extension was carried out using the primer (the coding sequence corresponding to the primer is underlined in C) labeled with [γ-32P]ATP as described under "Materials and Methods." The tentative initiation site of transcription was represented by an asterisk. C, DNA sequence of the promoter region of the ntp operon. The sequence corresponding to the primer used for primer extension was underlined. The putative Shine-Dalgarno (SD) sequence (boldface) and −10 and −35 boxes (overlined) were represented.
and the same initiation spots were observed.\(^2\) At this time, it is important to determine the unique transcription initiation site among these three spots (G, T, or C), but we tentatively assigned G as the site, located 40 bp upstream of the ntpF gene.

The amount of primer extension products changed drastically in parallel with the activities of the Na\(^+\)-ATPase in the membranes under various growth conditions. Amount of the products was limited in the cells grown on the Na\(^+\)-limited medium (Fig. 5B, lane 3). However, the amount of the spots increased at high Na\(^+\) concentration (Fig. 5B, lane 2), and it further increased in high Na\(^+\) and high pH medium (Fig. 5B, lane 1). In a previous paper (19), we performed the Northern blotting analysis with the probes corresponding to several parts from ntpA to ntpD genes and total RNA extract prepared from the cells cultured in these media. The increases in the mRNA for the ntp operon were in parallel with those observed here by the primer extension. Western blotting with anti-\(\alpha\)-subunit serum also revealed that the increase in the amount of the A subunit of the Na\(^+\)-ATPase in cell lysates was in parallel with the increases of transcripts in these conditions (19). These results suggest that de novo synthesis of Na\(^+\)-ATPase is regulated at the transcriptional level by sodium ions.

**Intracellular Na\(^+\) Regulates Transcription of the Na\(^+\)-ATPase Operon**—We have speculated that an increase in the internal Na\(^+\) concentration ([Na\(^+\)]\(_{\text{in}}\)) triggers stimulation of Na\(^+\)-ATPase biosynthesis (18). Fig. 6 shows the effect of monensin, a Na\(^+\)/H\(^+\) exchanging ionophore, on the amounts of the A plus B subunits of the Na\(^+\)-ATPase at the various concentrations of the external Na\(^+\) ([Na\(^+\)]\(_{\text{out}}\)). In the absence of monensin, the amounts of these subunits increased in proportion with the increase in [Na\(^+\)]\(_{\text{out}}\) and, at 1 mM NaCl, the amounts of these subunits increased up to about 15-fold (Fig. 6, open circles). When the membranes were rendered permeable to sodium ions by monensin, the amounts of the A plus B subunits increased by 6-fold of that of the untreated cells even in Na\(^+\)-limited KNY medium; the contaminated Na\(^+\) was less than 1 mM. The amounts of these subunits in the presence of monensin were also affected by [Na\(^+\)]\(_{\text{out}}\) but they remarkably increased at the lower concentrations of [Na\(^+\)]\(_{\text{out}}\). At 100 mM NaCl, the amounts of these subunits increased by 22-fold of those of the cells in KNY medium, and the increase in the amounts of Na\(^+\)-ATPase was finally saturated (Fig. 6, closed circles). Gramicidin D, which renders the cell membrane unselectively permeable to monovalent cations such as H\(^+\), Na\(^+\), or K\(^+\), brought about the same effect as monensin. Gramicidin D (5 \(\mu\)g/ml) enhanced the amounts of the A plus B subunits only 5-fold in Na\(^+\)-depleted medium. However, in the presence of 50 mM Na\(^+\), gramicidin D enhanced the amounts of A and B subunits by 17-fold (data not shown). In parallel with the amounts of these subunits, the Na\(^+\)-stimulated ATPase activity of the membranes of the cells grown in NaTY medium and in the same medium containing gramicidin D were 0.11 and 0.42 \(\mu\)moles/min/mg of protein, respectively. Although the proton gradient of growing cells was dissipated by the ionophore, the increase in the amounts of Na\(^+\)-ATPase was limited without addition of NaCl, suggesting that the effect of the size of the proton gradient was negligible for the induction of Na\(^+\)-ATPase. Thus, the amount of Na\(^+\)-ATPase is probably regulated by [Na\(^+\)]\(_{\text{in}}\). The effect of monensin on the amounts of mRNA for the ntp operon was also examined by Northern blotting (Fig. 7). In this experiment, the probe, a 2.5-kilobase HindIII-HindIII DNA fragment that corresponds to the ntpA to ntpD genes in the ntp operon (Fig. 7, shaded box) was used, and the total RNA fraction was prepared from the cells grown in four different media: KNY medium, KNY medium supplemented with 5 \(\mu\)g/ml monensin, KNY medium supplemented with 100 mM NaCl, and KNY medium supplemented with both 100 mM NaCl and monensin. The amount of mRNA for the ntp operon was negligible in KNY medium (Fig. 5B, lane 1), and it was slightly increased by supplementation with NaCl (lane 2) or monensin (lane 3). Then, the mRNA of this operon was increased remarkably by supplementation with both NaCl and monensin. The relative amount of mRNA in these fractions corresponded to the values of the amounts of the A and B subunits in the cells grown in the same media as shown in Fig. 6. All of these results suggest that the Na\(^+\)-ATPase level of *E. hirae* is regulated by [Na\(^+\)]\(_{\text{in}}\). Transcription of the ntp operon is stimulated by an increase in [Na\(^+\)]\(_{\text{in}}\) as the signal.

**DISCUSSION**

Although the ion specificity of *E. hirae* Na\(^+\)-ATPase has been reported in a previous paper (8), the data were based on the characteristics of the enzyme activity with Na\(^+\)-stimulated ATP hydrolytic activity that was too low. Ion specificity of the ATP hydrolytic activity by this enzyme was reexamined. The ATP hydrolytic activity was stimulated by not only NaCl but LiCl; the apparent \(K_m\) values for Na\(^+\) and Li\(^+\) were about 3 and 2 mM, respectively. KCl, CsCl, and ammonium chloride did not stimulate activity. As the sodium-pumping activity via the Na\(^+\)-ATPase in whole cells was also induced by supplementa-

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\(^2\) T. Murata, I. Yamato, K. Igarashi, and Y. Kakinuma, unpublished results.
The nhaA gene encodes a 27-kDa hydrophilic protein that is highly expressed (22) in high pH. The E. hirae cells grown in media containing 0.5 M NaCl were transferred to a Na+-limited medium. The amount of the A and B subunits of Na+-ATPase decreased by one-half of the original level within 1 h after the medium change. In this period, the cell mass increased by about 2-fold. Therefore, assuming that E. hirae ceased to synthesize the enzyme in Na+-limited medium, the decrease in Na+-ATPase level may be explained by a dilution of the enzyme during cell division, although further investigation is required.

It is noteworthy that the Escherichia coli nhaA gene encoding the Na+/H+ antiporter is highly expressed in high concentrations of Na+ or Li+ and in alkaline medium (30), suggesting that the nhaA gene may be regulated by the internal Na+ content (5, 31). The nhaR gene, downstream of the nhaA gene, has been reported as a positive regulator for expression of the nhaA gene (32); it would be interesting to investigate whether NhaR is a Na+-sensor. There are several palindromic sequences in the promoter region of the ntp operon (12), which may be the regulatory element(s) for the expression of this operon. It is hard to expect that sodium ions directly interact with the promoter region of the ntp operon. Transcription of the Na+-ATPase operon is probably regulated by a Na+/H+ antiporter (protein)-mediated system. Just upstream of the ntp operon, there is the ntpR gene, which encodes a 27-kDa hydrophilic protein having a putative helix-turn-helix motif. At first, we expected this gene to be the regulatory gene for the ntp operon and knocked it out by gene disruption. However, the ntpR-disrupted strain did not show any difference in induction of the Na+-ATPase.3 Our attempt to detect a sodium-specific response in a gel retardation assay with cell lysates and the plasmid harboring the DNA region upstream of the operon has been so far unsuccessful. We will isolate regulatory mutants of the operon induction as another approach for identifying the regulatory protein.

Recently it was pointed out that transcription of the napA gene, the Na+/H+ antiporter gene of E. hirae, may be stimulated by an increase in [Na+] out or [Li+] out (33). The Na+-ATPase-defective mutant Nak1 (34) did grow in NaTY medium containing more than 0.5 M NaCl at low pH (data not shown). These results suggest the possibility that induction of Na+-ATPase may be compensated for by an elevation in the antiporter activity under this condition. Thus, the internal Na+ concentration of E. hirae is probably regulated by an interplay between these two extrusion systems at the transcriptional level.

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