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Characteristics of ATPases Present in Everted Membrane Vesicles of *Helicobacter pylori*

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Everted membrane vesicles of *Helicobacter pylori* were prepared and the membrane-resided ATPases were characterized. For comparison, *Escherichia coli* membrane ATPases and hog gastric mucosal H,K-ATPase were employed. ATPase assay revealed that the composite enzyme pool was relatively low in specific activities, below 1/10 times than that found in *E. coli*. According to their inhibitory specificities, most of the ATPase pool appeared to belong to the P-type ATPase, sensitive to vanadate but not to azide. The enzyme pool was extraordinarily resistant against treatment by N,N'-dicyclohexylcarbodiimide (DCCD). Certain monovalent cations, e.g., K^+ or NH_4^+ stimulated the whole enzyme pool only in the presence of Mg^{2+}. On the contrary, Ni^{2+} and Zn^{2+} increased enzyme activity rather effectively without the aid of Mg^{2+}. Under a defined condition employed, *H. pylori* cells could retain the membrane ATPase pool to the extent of 17% at pH 3.2. Moreover, its activity was most stable in acidic conditions (pH 5.4~6.4). However, cytoplasmic or peripheral ATPase pools were hardly detected under acidity (below pH 4.6).

After its first discovery by Marshall *et al.* in 1982 (19, 21), the etiological significance of *Helicobacter pylori* for diseases occasioned in human gastrointestinal tracts has become a matter of world-wide concern and interest (5, 6, 13, 14, 25). To date clinical evidence strongly suggests that this organism, as a causative agent, is closely associated with the recurrence or occurrence of gastritis and peptic ulcers (27). Thus, the eradication of *H. pylori* is now recommened for patients with such diseases, along with acid suppression from gastric parietal cells (6, 17).

H. pylori is a gram-negative microaerobic *bacillus* which is spiral rod and flagellated. This bacterium is exclusively found in gastric biopsy specimens (26). *H. pylori* in particular has the unique ability of being able to survive in both acidic and neutral environments, suggesting that this organism may belong to facultative acidophile. However, there is still controversy as to whether the cellular responsiveness to external pH is opportunistic. This bacterium secretes considerable amounts of urease, supposed to facilitate the cell's survival against ambient acidity. However, this function itself can not sufficiently contribute to neutralize intracellular pH (10, 11, 12, 20). The cells perhaps need another fundamentally active machinary to enable them to adapt more directly to acid for survival and /or growth. In other words, it requires a potential system in order to control proton concentrations across the cell membrane.

Proton translocating ATPases are among the most fundamental enzymes in nature (23). In general, their roles in energy transduction and pH homeostasis are uniformly crucial throughout living systems (24). These enzymes are membrane-resident, and can be classified into three groups; F-type, P-type and V-type ATPases. Of these, only F-type ATPases function to synthesize ATP, suggesting that this family may play an essential role in living systems (7). V-type ATPases conflict with F-type ATPases and are not involved in the ATP synthesis but mediate vacuolar acidification (22). P-type ATPases can be distinguished from the above enzyme types by their formation of phosphorylated intermediates during turn over (E_1-E_2) (27).

It is suggested that bacterial P-type ATPases are involved in cell turgor maintenance, pH homeostasis, osmoregulation or signal mediation (16). Unlike the acidophilic adaptibility against constant acid, the role of these enzymes is thought to be pivotal when encountering accidental acid for maintenance of cell's existence (1). Iterative change of pH in human body occurs only in the stomach, occasioned by nutrient-mediated acid secretion from gastric parietal cells. It is interesting that *H. pylori* survives this hostile environment as an ecological niche. Accordingly, its counteractive function against such a random acidity is thought to be as a rational target for eradication of *H. pylori*.

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Recently, a number of gastric proton pump inhibitors such as benzimidazole derivatives, have shown promising effectiveness in eradicating *H. pylori*, provided that these are administered along with antibiotics (2). Unfortunately, the increased gastric pH would give rise to reinfection by this bacterium (30). On the other hand, studies have reported these days about *in vitro* antibacterial activity of those inhibitors against *H. pylori*, where a number of enzymes including proton pumps are known to be inhibited (3, 15). Arguments about their molecular effectiveness against *H. pylori* are not yet conclusive. Regarding the pumping machinery, no noticeable studies have been reported.

In this study, the enzyme types and cation specificities of membrane ATPases in *H. pylori* were investigated using ATPase inhibitors. Also, the effect of extracellular pH on the expression of these enzymes was examined by using *H. pylori* intact cell system, and compared it with observation of soluble ATPases originated from cytoplasm or periplasm.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

A strain of *H. pylori* was kindly provided from CURE at the Department of Medicine, UCLA, Los Angeles, CA (U.S.A.). *E. coli* HB101 was generously donated by the Lipid Research Laboratory, WLA/VA Medical Center & UCLA, Los Angeles, CA (U.S.A.). *E. coli* No. 20 was a stocked strain in our laboratory. Primary culture of *H. pylori* was undertaken by inoculating cells onto petri plates containing brain heart infusion agar and 10% virus-free horse serum, pH 6.8 (BHS), followed by incubation at 37°C for 2~3 days using an incubator (Heraeus, type BB6060) controlled in air contents (5% O_2-10% CO_2-85% N_2). After incubation, cell colonies were harvested, and inoculated onto 100 ml liquid BHS in Erlenmyer flask. Following cultivation by reciprocal shaking for 2 days with the above air composition, the culture broth (O.D. at 660 nm, 0.15~0.25) was centrifuged, and the cell pastes were stored at -80°C. Cells of *E. coli* were grown overnight at 37°C in LB medium, pH 7.0, and centrifuged. The resulted cell pastes were stored at -80°C.

Preparation of Everted Membrane Vesicles

Everted vesicles were prepared as described by Sieber *et al.* (28). All of the following steps were performed for both membranes of *H. pylori* and *E. coli*. Procedures were carried out on ice unless stated otherwise (for centrifugation, at 4°C). Frozen cells were thawed, washed once with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Tris buffer containing 20 mM $MgCl_2$, pH 7.4, and centrifuged at 9,000 g for 10 min. Cells were then resuspended in 50 mM HEPES-Tris buffer, pH 7.4, containing 1 mM 1,4-dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol. Cell suspension was passed 3 times through French Press (16,000 psi; Avestin, Canada, Model B3042) at 4°C. If necessary, cells were resuspended in 20 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM [ethylenedinitrilo] tetraacetic acid (EDTA) and 0.1 mg lysozyme/ml, and incubated for 30 min for sonication. Intact cells were removed by centrifugation (9,000 g for 10 min), and the resulted supernatants were ultracentrifuged at 190,000 g for 1 h. After centrifugation, the supernatant containing cytoplasmic and peripheral proteins was directly used for soluble ATPase assay. The membrane pellet was washed once by resuspending it in 1 mM HEPES-Tris buffer (pH 7.4) containing 3 mM EDTA and 0.5 mM PMSF, and centrifuged (190,000 g for 1 h). The precipitated pellet was suspended in 50 mM Tris-HCl, pH 7.4. This vesicle prepared was either directly used for experiments, or was on ice for several days. In the latter case, most of the total ATPase pool was retained. For prolonged storage, the vesicle pellet could either be lyophilized or be stored at -80°C. Gastric H,K-ATPase (90% purity) was an authentic gift from Dr. Sachs at Membrane Biology Laboratory, WLA/VA, UCLA, Los Angeles, CA (U.S.A.).

Expression of Membrane ATPases from Intact Cells of *H. pylori*

Procedures were carried out on ice unless stated otherwise (for centrifugation, at 4°C). Frozen cells were thawed, suspended in 50 mM HEPES-Tris buffer containing 10 mM $MgCl_2$, pH 7.4, and centrifuged. Portions of the cell pellet were carefully resuspended to give final turbidity of 1.0 (O.D. unit at 660 nm) in a reaction system consisting of 1% glucose, 20 mM $MgCl_2$, 25 mM urea, 0.05 mM $NiCl_2$, 0.015 mM ionomycin and 20 mM KPO_4 (pH range, 3.2~9.5). Following the preparation of intact cell systems, incubation was performed at 37°C for 2.5 h in an incubator controlled composition of air as described before. After incubation, cells were collected by centrifugation (4,000 g for 10 min), washed once by resuspending in 50 mM HEPES-Tris buffer, pH 7.4, and centrifuged. Cells were then resuspended in 50 mM HEPES-Tris buffer, pH 7.4 containing 1 mM DTT, 0.5 mM PMSF, 10% glycerol, and disrupted by passing them 3 times through a French Press (16,000 psi at 4°C). Cell debris was discarded by centrifugation (9,000 g for 10 min) and centrifuged again (190,000 g for 1 h). The resulting supernatant was used for soluble ATPase assay. Precipitated membrane pellets were carefully suspended in a buffer consisting of 0.25 M sucrose, 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) and 20 mM Tris-HCl, pH 7.4 using a homogenizer, and stored either on ice overnight or at -80°C before use in membrane ATPase assay.

ATPase Assay

Typically, the reaction mixture consisted of membranes (1~200 μg of proteins), 2 mM $MgCl_2$, 0.25 M sucrose and 20 mM Tris-HCl buffer, pH 7.4 in a final volume of 1 ml. Enzyme reactions were initiated by the addition of 2 μmoles of ATP at 37°C. Inhibitors were preincubated with enzyme as follows: For azide, at 37°C for 10 min; N,N'-dicyclohexylcarbodiimide (DCCD), at 37°C for 20 min, and for vanadate, at 37°C for 2 min, respectively. The enzyme reaction was stopped by adding 1 ml of ice-cold acid molybdate (4.5% ammonium molybdate-60% perchloric acid=4 : 1, v/v), followed by extraction of molybdo-phosphate complex using 2.5 ml of n-buthylacetate with vigorous vortex (32). The clearer upper phase was obtained by centrifugation at 1,000 rpm for 5 min (Dupon Inc., U.S.A.). Enzyme activity was determined by measuring the absorbance at 320 nm using Gilford spectrophotometer Model 2530-35 (ε_{320}=7,500 $M^{-1}cm^{-1}$). Activity was defined as nano moles of ATP hydrolyzed per milligram per minute.

Protein Determination

Protein concentration was measured by the method of Lowry *et al.* (18) using bovine serum albumin as a standard.

Chemicals

Sodium azide and ammonium molybdate were purchased from Fisher Scientific. DCCD and 1-O-n-octyl-β-D-glucopyranosid (n-octylglucosid) were obtained from Aldrich and Boehringer Mannheim GmbH, respectively. All other products were from Sigma.

RESULTS

Determination of Membrane ATPase Activities

ATPase activities were determined using everted membrane vesicles. As shown in Table 1, *H. pylori* membranes exhibited unusually poor enzyme activity, below 1/10 times than that found in *E. coli* membranes. To ascertain whether the overall low in the specific activity was due to problems in the enzyme-substrate interaction, those *H. pylori* membranes were loosened by mild treatment with nonionic detergents, and tested again. Despite this effort, the increase of specific ATPase activity was not considerable. Treatment with chelating agents such as EDTA or EGTA resulted in no discernible change in the ATPase activity. However, 1,2-cyclohexanediaminetetraacetic acid (CDTA) depressed enzyme activity by about 50%, indicating the involvement of divalent cation, i.e., Mg^{2+} for enzyme catalysis (Table 2). These observations suggest that *H. pylori* cells probably express far smaller amounts of membrane ATPases compared to that of microorganisms in general. In order to clarify the significance of extraordinarily low ATPase pools in *H. pylori* membranes, the nature of membrane ATPases were preferentially identified using ATPase inhibitors.

Identification of Membrane ATPases of *H. pylori*

An experiment to ascertain F-type ATPase (EC 3. 6. 1. 3) was carried out using azide, a compound known to inhibit specifically F_1-domain of this enzyme (8). For comparison, membranes of *E. coli* and gastric H,K-ATPase (a P-type ATPase of the plasma membrane) (28) were employed. It is known that apparent membrane ATPase activity in *E. coli* is exclusively derived from F-type ATPase (8). In Fig. 1 inhibitory slopes for *E. coli* ATPase pool were seen to cross at 0.06 mM azide, suggesting the involvement of alternative enzyme types other than F-type ATPase. On the other hand, gastric H,K-ATPase activity was scarcely inhibited by azide treatment. Since the H,K-ATPase employed was thought to be somewhat contaminated by mitochondrial F-type ATPase, the resulting data was anticipated. By contrast, the effect of vanadate for these enzymes was almost completely opposed to the azide effect (Fig. 2). Since vanadate inhibits only P-type ATPases, it was reasonable observation that gastric H,K-ATPase lost over 90% of its activity with only 0.016 mM vanadate. *E. coli* ATPase pool, however, was not significantly decreased by this compound up to 0.2 mM. These findings demonstrate that more than 80% of all *E. coli* ATPases consist of F-type ATPase, and of course, gastric H,K-ATPase is a P-type ATPase. In accordance with the above methodology, a time course experiment for ATP hydrolysis with everted vesicles of *H. pylori* was performed to determine its ATPase pool using azide and vanadate. As shown in Fig. 3, the rate of ATP hydrolysis was slightly decreased by 0.1 mM azide, but residual activity was almost completely depressed by

Table 1. Comparison of specfic activities of membrane ATPases between *H. pylori* and *E. coli*.

Membrane	Condition	ATPase activity (*n*mole/mg/min)
H. pylori	French press	13.3
E. coli No. 20	〃	240.0
E. coli HB 101	〃	130.2

Table 2. Effect of some chelators and detergents on the ATPase activity present in everted membrane vesicles of *H. pylori*.

Compound	Conc.	ATPase activity (*n*moles/mg/min)
None	–	13.3
EDTA	3 mM	12.0
EGTA	3 mM	11.2
CDTA	3 mM	7.6
Triton X-100	0.01%	16.1
n-Octylglucosid	0.01%	28.8

French pressed membrane vesicles from *H. pylori* were preincubated with chelators (3 mM) for 1 h on ice, or with detergents (0.01%) for 5 min at 37°C prior to the addition of ATP.

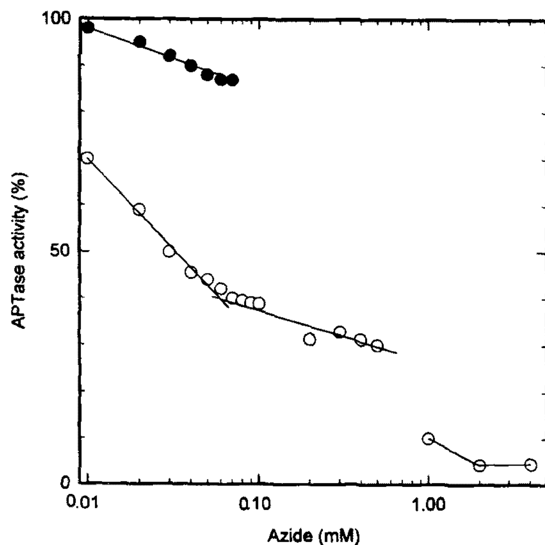

Fig. 1. Effect of azide on the membrane ATPase activities from *E. coli* and hog gastric mucosa.

70 μg of everted vesicles from *E. coli* (○) and 2 μg of cytoplasmic-side-out vesicles (●) (homogeneous in H,K-ATPase) from hog gastric mucosa were preincubated with or without various concentrations of azide for 10 min at 37°C before the addition of ATP. Activity is presented by percent ratio relative to that found in the absence of azide.

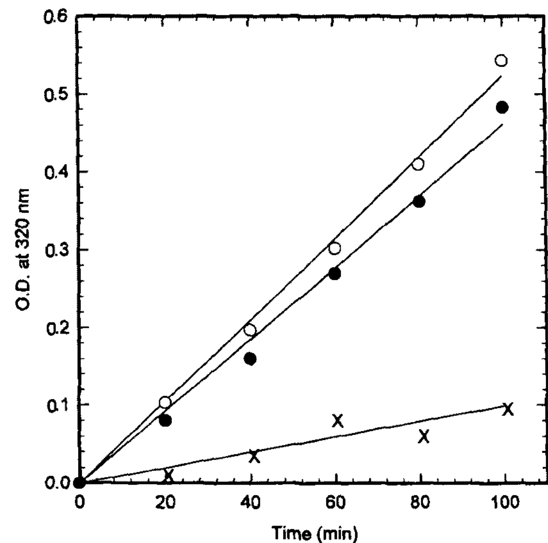

Fig. 3. Time course inhibition of *H. pylori* ATPase pool by ATPase inhibitors.

100 μg of membrane vesicles from *H. pylori* were incubated in the absence (○) and presence of 0.05 mM azide (●) or, in addition, 0.1 mM vanadate (×) before the addition of ATP. For details, refer to Materials and Methods. Time course hydrolysis of ATP was monitored discontinuously.

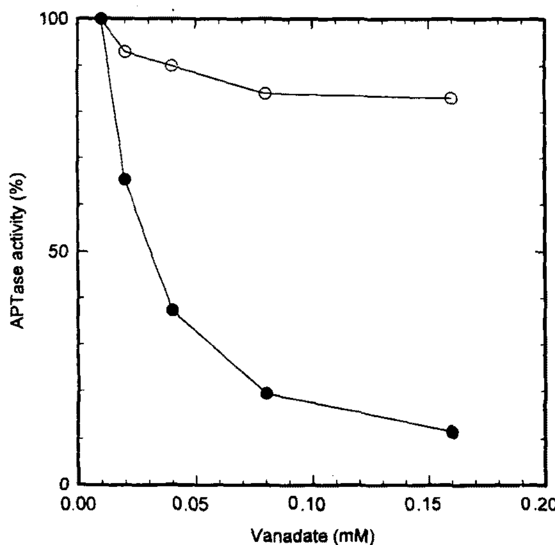

Fig. 2. Effect of vanadate on the membrane ATPase activities from *E. coli* and hog gastric mucosa.

Enzymes were treated with various concentrations of vanadate for 2 min at 37°C prior to the addition of ATP. For other conditions, see Fig. 1. Symbols used: (○), *E. coli* ATPases; (●), hog gastric H,K-ATPase.

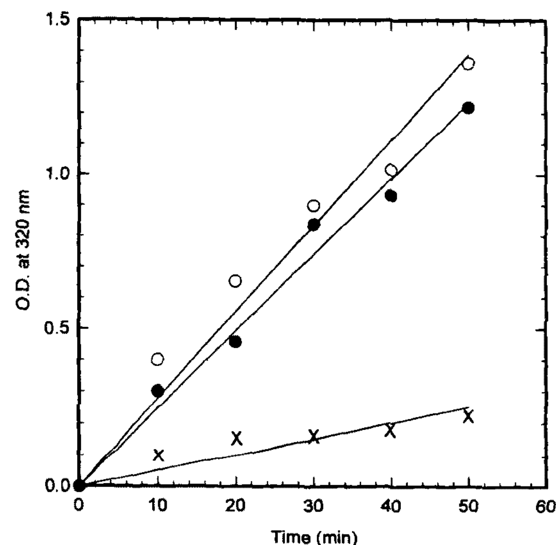

Fig. 4. Time course inhibition of hog gastric H,K-ATPase activity by ATPase inhibitors.

2 μg of vesicled enzyme was used. For experimental conditions and symbols used, see Fig. 3.

additional treatment with 0.1 mM vanadate. This data suggest that *H. pylori* expresses low level of F-type ATPase. In fact, a similar result was observed from gastric H,K-ATPase (Fig. 4). As a further assessment of the property of *H. pylori* ATPases, the effect of DCCD on the enzyme activity was compared with controls. As can be seen in Fig. 5, the *H. pylori* ATPase pool was resistant by more than 1 or 2 folds of logarithmic magnitude against DCCD relative to those found in the gastric H,K-

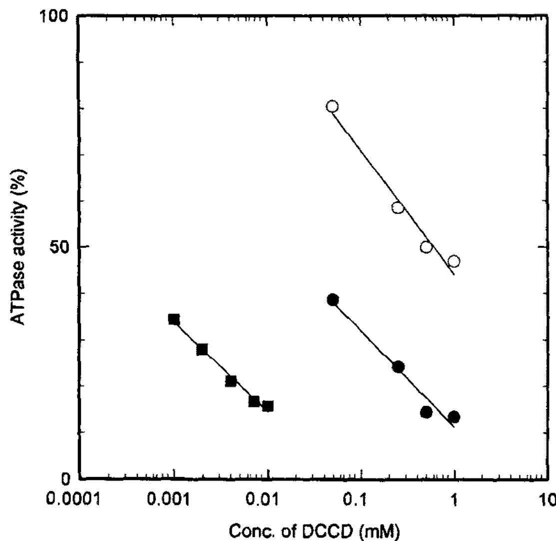

Fig. 5. Comparison of DCCD effect between *H. pylori* ATPase pool and control enzymes.
Membrane vesicles were pretreated with DCCD in the range of 0~1 mM (dissolved in dimethylformamide) before the addition of ATP. Activity is presented by percent ratio relative to that found in the absence of DCCD. Symbols used: (○), *H. pylori* ATPase; (●), gastric H,K-ATPase; (■), *E. coli* ATPase.

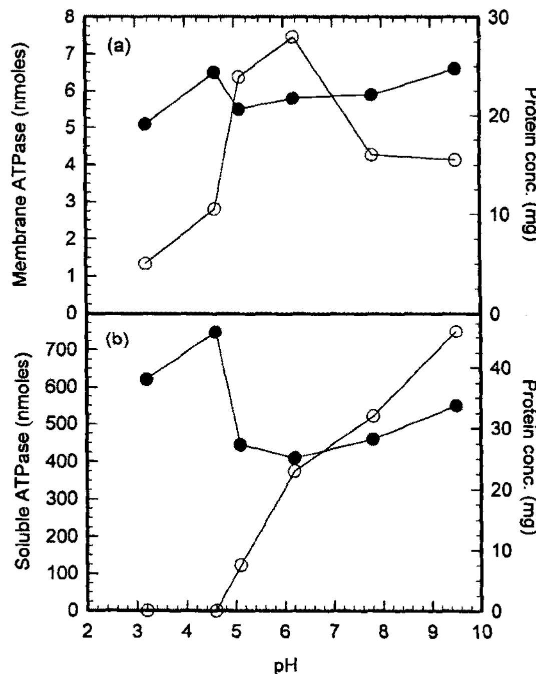

Fig. 6. pH Dependence of *H. pylori* ATPases in the intact cell system.
Detailed procedure for membrane preparations was described in Materials and Methods. (a), Dependence of membrand-bound ATPase pool against medium pH. (b), Dependence of soluble ATPase pool against medium pH. Symbols used: (○), total enzyme activity; (●), protein concentration.

ATPase or *E. coli* ATPase pool, respectively. It was interesting though that all the inhibitory slopes with DCCD were similar.

Effect of Cations on the Membrane ATPase Pool of *H. pylori*

Under the apparent observation that much of the ATPase pool of *H. pylori* was composed of certain P-type ATPases, the cation specificities of these enzymes were investigated. In the presence of Mg^{2+}, certain monovalent cations such as K^+ or NH_4^+ were the most effective cations in terms of increasing total enzyme activity, but they ceased increasing at 2-fold. However, where the reaction system was deprived of Mg^{2+}, Ni^{2+} or Zn^{2+} accelerated its activity to some extent (Table 3).

Effect of Extracellular pH on the Membrane ATPase Pool of *H. pylori* Intact Cells

As explained before, *H. pylori* has the unique property of being able to produce extraordinarily high amounts of extracellular urease. In order to understand the significance of urease production in this organism, an experiment to assess the expression of membrane-bound ATPases was undertaken at different pHs using the intact cell system containing urea and Ni^{2+} (refer Materials and Methods). After incubation for 2.5 h at 37°C, the volume of cell pellets did not change when the extracellular pH was below than 6.4. Over this pH, however, the pellet volume was increased by up to 3-fold and the increase in the volume

Table 3. Effect of cations on the ATPase pool in everted membrane vesicles of *H. pylori*.

Cation	Conc. (mM)	ATPase activity (*n*moles/mg/min)	
		with Mg^{2+}	without Mg^{2+}
None	–	12.3	3.7
$BaCl_2$	0.05	13	7.7
$CaCl_2$	0.05	10.3	ND
$CuSO_4$	0.05	13.3	11
$FeSO_4$	0.05	15.7	ND
H_2MoO_4	0.05	10	ND
KCl	20	25	22.3
$MgCl_2$	20	–	102.3
$MnCl_2$	0.05	12.3	11.3
NaCl	20	ND	11.3
NH_4Cl	50	25.3	28
$NiCl_2$	0.05	11.7	47
$ZnSO_4$	0.05	10.7	22.3

Membrane suspension (100 μg protein in 20 mM Tris-HCl, pH 7.4) containing 0.25 M sucrose, 0.05 mM azide and cation was preincubated at 37°C for 20 min before the addition of ATP. ATPase reaction was carried out in the presence or absence of 2 mM $MgCl_2$ for 10 min, and the amount of Mo-phosphate complex produced was expressed by nano moles (refer to Materials and Methods for details). In order to eliminate the residual magnesium ion membrane vesicles were treated with 0.1 mM EGTA and dialyzed before use. 1 μg of ionophores (nigericin or ionomycin) were supplemented for the transport of mono or divalent cations, respectively. ND, not determined.

was proportional to the pH increase (data not shown). In relation to ATPase expression, the cytoplasmic or peripheral ATPase activities showed no activity at low pH (pH$\leqq$4.6). Whereas, the membrane ATPase pool was retained to a certain degree until pH 3.2, known as marginal acidity for *H. pylori* survival. Furthermore, its maximal expression was also observed in acidic conditions (Fig. 6). It was noteworthy that *H. pylori* intact cells could not maintain soluble ATPase but membrane ATPase pool at pH range of 3.2 to 4.6.

DISCUSSION

In order to clarify the constituent ATPases present in cell-membrane of *H. pylori*, studies were carried out in comparisons with those from *E. coli* and gastric parietal cells as standards for F-type and P-type ATPases, respectively. Most of the ATPase pool in *H. pylori* membranes were insensitive against azide treatment, although the corresponding pool in permeabilized cells of *H. pylori* were found to be sensitive (4). However, by raising the azide concentration (up to 5 mM), the activity pool was reduced to the extent of 70% (data not shown). Similar effects are shown in Fig. 1, and we suppose that these phenomena were caused by F-type ATPase tolerant against a limited concentration of azide. On the other hand, the *H. pylori* enzyme pool was readily inhibited by vanadate (Fig. 3), which is known as a specfic inhibitor for the P-type ATPase family. It was interesting that *H. pylori* ATPases were extreamely resistant against treatment by DCCD. As indicated in Fig. 5, only 50% of the whole activity was inhibited by 0.5 mM DCCD. But, under the same conditions, not only *E. coli* ATPases but also gastric H, K-ATPase were almost completely inhibited. As contrasted with binding affinity of DCCD for *E. coli* Kdp-ATPase (28), the resistance of membrane ATPase pool of *H. pylori* was exceptional. This property suggests that *H. pylori* ATPases in general would contain unique structures resistant to DCCD. Following the characteristic properties observed by ATPase inhibitors, it was strongly suggested that the membrane ATPase pool of *H. pylori* was exclusively composed of P-type ATPases. This in turn indicates the negligible exhibition of F-type ATPase activity. Accordingly, it seemed most of the ATP necessary for cell physiology in this organism would not be suplied by the machinery of oxidative phosphorylation, but perhaps depend upon other sources.

In spite of the additional presence of ionophores (i.e., ionomycin or nigericin), however, there was no significant change in the cation-mediated enzyme activity (data not shown). Unfortunately, regardless of effort to increase the specific activity of the membrane-ATPase pool in everted vesicles of *H. pylori*, it could not be successfully accomplished (Table 1). Based on the effect of mono or divalent cations, it was thought that a number of P-type ATPases would exist in *H. pylori* (Table 3). It is presumed that a certain proton pump would involve in Ni^{2+} uptake, pertaining to the maintenance of cellular requirement for urease biosynthesis.

An interesting property of *H. pylori* was found by using intact cell system of this bacterium (Fig. 6a). Under the condition employed, its membrane ATPase pool was considerably stable against acidic environment (pH$\geqq$3.2). And, optimal pH for this enzyme stability was also observed at pH range of 5~6. It is unclear though whether this result was due to the extra presence of urea and Ni^{2+}. Nevertheless, it seems that this unusual property would be essential for counteractive nature of *H. pylori* against acid. On the contrary, cytosolic or peripheral ATPase activities were not detected below pH 4.6, but proportionally increased by increasing the extracellular pH by unknown reason (Fig. 6b). Another notable feature of *H. pylori* was seen during incubation of the intact cells under limited concentration of oxygen gas (5%). After all, individual cells were clotted to become bolls (2~3 mm in diameters), accompanied with considerable increase in the volume of cell pellets over neutral pH (data not shown). This *in vitro* phenomenon would be related to cell's colonization *in vivo*.

Bacterial P-type ATPases in particular are not fully investigated (9), because of difficulties in accessing these enzymes, for which an high background of F-type ATPase usually pertains. Nevertheless, their role is thought to be essential against accidental acids (1). Although various reports have been dealing with ATPases in *H. pylori*, unexpected several findings in this study come into question as follows: (a); The absence of enough F-type ATPase, theoretically required, (b); The nature of P-type ATPases that are barely assessable, and (C); The significance of maintenance of membrane-bound ATPases activity against acidic environment. Further investigations will be pursued to solve these problems.

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