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Synergistic Inhibition of Membrane ATPase and Cell Growth of *Helicobacter pylori* by ATPase Inhibitors

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Abstract *Helicobacter pylori* were found to be resistant to azide but sensitive to vanadate, suggesting that defect in the P-type ATPase activity rather than F-type ATPase would be lethal to cell survival or growth. To elucidate the relationship between this enzyme inhibition and *H. pylori* death, we determined the effect of omeprazole (OMP) plus vanadate on enzyme activity and cell growth. The minimum inhibitory concentration (MIC; ca. 0.8 μmol/disk) of vanadate for *H. pylori* growth was lowered over 10-fold with the aid of OMP, whereby its inhibitory potential toward the P-type ATPase activity was diametrically increased. Alternatively, we found that this enzyme activity was essential for active transport in *H. pylori*. From these observations, we strongly suggest that the immediate cause of the growth inhibition of *H. pylori* cells with OMP and/or vanadate might be defective in the cell's active transport due to the lack of P-type ATPase activity. From the spectral data with circular dichroism (CD) spectroscopy, we found that activated OMP (OAS) at concentration below MIC did not disrupt helical structures of membrane proteins. Separately, we determined the cytopathic effect of OAS by SDS-PAGE, indicating the change in the production of cytoplasmic protein but not cell membrane.

Key words: *Helicobacter pylori*, P-type ATPase, omeprazole, ATPase inhibitors

Helicobacter pylori is a parasitic gram-negative bacterium spiral with lophotrichated flagella [24]. *H. pylori* inhabit human stomach as an ecological niche [13], however, its free-living form in nature is yet to be identified. This in turn implies that *H. pylori* are obligatory parasites. Infectious gastric diseases (e.g., gastritis or peptic ulcers) are extremely incurable, often followed by chronicity or recurrence [25]. In recent years, such undesirable outcomes of peculiar properties of *H. pylori* have been explained as follows: This organism can deeply penetrate the gastric mucous layer, interact persistently with the host cells, and thereby optimize such a hostile environment for survival and growth [7]. To colonize, nutrients must be amply supplied by devastating the nearby host cells. In fact, a prolonged infection with *H. pylori*, which liberates harmful cytotoxic or highly reactive materials [3], promotes various types of cell aberration and/or atrophies that are often associated with gastric cancer [23, 28]. Despite extensive studies, the origin of *H. pylori* infection is still obscure. Accordingly, the cure for patients with such gastric diseases is currently viewed in clinical medicine as related to the eradication of *H. pylori* [14, 20]. Unfortunately, no promising results have yet been attained from extensive studies in chemotherapy, and emerging drug resistances have induced these diseases to become more complicated [1, 30]. The eradication of *H. pylori* is, therefore, imperative for patients suffering from gastric diseases.

In practice, no predicted probability has yet been established concerning the frequency of compounds that are active *in vitro* that can also be effective *in vivo* [26]. Until now, regimens for curing such complex diseases associated with *H. pylori* are all but dual or triple therapies with the aid of an antacid [16]. Of the antacids, a substituted family of benzimidazoles [e.g., omeprazole (OMP)] is widely used along with antibiotics [17, 19]. Interestingly, these compounds exhibit antibacterial activity, particularly against *H. pylori*. Studies have, therefore, been focused on accessing their action mechanism, however, nothing has yet been clarified [5, 15]. Recently, it was found that most of the ATPase activity in the *H. pylori* membrane were sensitive to vanadate which indicated that the enzyme was P-type ATPase. In addition, the enzyme inhibition caused by OMP or its activated form (OAS) was accompanied by the inhibition of both the peptide transport and growth inhibition of *H. pylori* cells. Under serum-free conditions, various amino acids have to be supplied for *H. pylori* growth as carbon or energy sources preferable to glucose [9, 10]. It has been suggested that the P-type ATPase activity in the *H. pylori* membrane is necessary for the active transport of essential nutrients. OMP seems to be an

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ideal tool for accessing the successful eradication of deeply colonized *H. pylori*, since it can readily penetrate and spread across the gastric mucous layer, and can even be found in the canaliculi of gastric parietal cells [6, 15], although it is still obscure as to whether or how this compound actually influences the infected *H. pylori* physiology.

To clarify the killing mechanism of OMP (OAS), we established membrane P-type ATPase of *H. pylori* as a target enzyme. In this paper, we investigated the synergistic effect of OMP with vanadate for cell growth, enzyme activity, and active transport. Separately, we studied whether OMP (OAS) could give rise to change in the membrane structure or protein synthesis in *H. pylori*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *H. pylori* strains used in this study are listed in Table 1, and included the American Type Culture Collection (Rockville, U.S.A.) and authentic strains. Other bacterial strains were obtained from the Korea Research Institute of Bioscience and Biotechnology, Korea. The frozen *H. pylori* cells were thawed, placed on an agar plate containing a brain heart infusion medium (Difco, Detroit, U.S.A.) and 5% virus-free horse (or bovine) serum, and grown microaerobically (in a jar with a gas mixture: 5% O_2, 10% CO_2, and 85% N_2) for 2 days at 37°C. After incubation, the colonies formed were harvested by scraping off the agar and then subjected to liquid cultivation. Subcultures were subsequently made by either transferring each of the vegetative cell crops or freezing them directly onto a liquid media (Erlenmeyer flask) containing the above medium contents. This cultivation was then followed by reciprocal shaking until reaching the late exponential growth phase before the emergence of coccoids. The culture broth was centrifuged for 10 min at 9,000 ×g and the resulting cell pastes were washed twice, concentrated with 10% glycerol and 5 mM Tris-HCl buffer (pH 7.4), and stored at below -80°C before use [32]. Other species were aerobically grown at 37°C using Luria medium (1% bacto peptone, 0.5% yeast extract, and 1% NaCl, pH 7.0). The resulting culture broth was centrifuged and the cell pellet was washed, concentrated, and stored as above.

Overall Fractionation of Intact Cells

H. pylori cells were resuspended in 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonate (HEPES)-Tris buffer (pH 7.4) to give an absorbance of 1.0 at 660 nm. This cell suspension was incubated for 10 min at 37°C in the presence or absence of 1 mM OMP. After incubation, the cells were washed by centrifugation (9,000 ×g, 10 min) and the supernatant was harvested (extracellular fraction). To obtain spheroplasts, the cells were exposed to thermal changes (freezing/thawing in distilled water) prior to lysozyme-EDTA treatment [33]. Following the disruption of the cell envelopes, the cells were separated by centrifugation (9,000 ×g, 10 min) from the supernatant. Both the outer membrane fraction and periplasmic fraction were isolated from this supernatant by ultracentrifugation (190,000 ×g, 1 h). The spheric cells were disrupted by a short exposure to ultrasonic waves (Sonics & Materials Inc., U.S.A.: VCX 400; frq. 20 KHz), followed by centrifugation (9,000 ×g, 10 min) to obtain a supernatant. Finally, the resulting supernatant was ultracentrifuged again to isolate the cytoplasmic and membrane fractions.

Preparation of Membrane Vesicles

Membrane vesicles were prepared from frozen cells as described previously [32]. The relative content of the everted (inside-out) vesicle was determined by comparing the ATP hydrolysis rate before and after treatment with either of chloroform or 1% *n*-octyl glucosid, which was constantly observed to be ca. 60%. The vesicles can be stored on ice for several days or deep-freezed for months without exhibiting any significant loss of activity.

ATPase Assay

An aliquot of freshly thawed membranes (~0.1 mg proteins) were mixed on ice in a reaction mixture containing 1 mM $MgCl_2$, 0.25 M sucrose, and 20 mM Tris-HCl, pH 7.4 (ST buffer) to give 0.9 ml. The selective suppression of F-type or P-type ATPase activities was carried out by treating the vesicles for 5 min at 37°C with 0.1 mM sodium azide and sodium ortho-vanadate. The enzyme reaction was initiated at 37°C by the addition of 0.1 ml of 1 mM ATP and terminated on ice by adding 1 ml of a stop solution (12% perchloric acid containing 3.6% ammonium molybdate). This mixture was then kept at room temperature for 10 min to exhibit a pale yellow color. Next, 2 ml of *n*-butylacetate was added, mixed vigorously, and centrifuged (1,000 rpm, 5 min). The colored molybdophosphate adducts in the upper phase were taken and the absorbance was read at 320 nm (ε_{320} = 7,500 $M^{-1}cm^{-1}$) [30]. Alternatively, ATPase activity can be assessed with reliable accuracy using a microtiter reader (E_{max} precision microplate reader; Molecular Device, U.S.A.). Briefly, 0.1 ml of the reaction mixture containing the membrane vesicles, 1 mM ATP, and the ST buffer was placed in a well (96-well microtiter plate) and incubated at 37°C on a stirrer (Titramax 1000; Heidolph, Germany). After the incubation, 0.1 ml of the above stop solution was added to the well and incubated for 10 min at room temperature. Twenty μl of 1% Fiske & Subbarow reducer (Sigma, St. Louis, U.S.A.) was then added to this mixture and the absorbance was read at 650 nm.

Circular Dichroism Spectroscopy

OMP was dissolved in acidic methanol (adjusted pH to be 1.5 by 0.1 N HCl) [4], and the resulting product, a cationic sulfenamide (OAS), was confirmed by TLC (toluene-ethylacetate = 1:2, $R_f \fallingdotseq 0.45$). OAS was added to the everted vesicle suspension (1 mg proteins/ml ST buffer) at various concentrations (0.1-1 mM) and incubated for 10 min at 37°C. The free OAS was completely removed by ultracentrifugation (19,000 ×g, 1 h), and the membrane pellet was resuspended in the ST buffer containing 1 mM EDTA. Circular dichroism (CD) spectra in the far-UV region (195-240 nm) were recorded on a Tasco J-710 spectropolarimeter (Japan) using a cuvette with a 1-cm light-path.

Nickel Transport Measurement

One-tenth mg of membrane vesicles was suspended in 0.5 ml of the ST buffer, mixed with 0.5 ml of 1 M $NiCl_2$, and diluted 10-fold with the ST buffer. The mixture was placed into a conical centrifuge tube (15-ml screw cap) and incubated overnight in an ice-bath with reciprocal shaking (75 rpm). After incubation, the tube was ultracentrifuged (190,000 ×g, 1 h) and the resulting membrane pellet was washed twice by ultrafiltration (polysulfone: 10,000 NMWC). The vesicle deposit on the filter was carefully harvested by a prechilled-ST buffer and used directly for nickel ion transport as follows: The transport system in a final volume of 10 ml was composed of Ni^{2+}-saturated vesicles (1 mg proteins), 1 mM $MgCl_2$, 1 mM ATP, and the ST buffer. This system was preincubated for 5 min at 37°C with ATPase inhibitors before the addition of ATP. Following the addition of 1 mM ATP, aliquots were taken at fixed time-intervals, immediately filtered, and washed as described above. The ATP-dependent export of Ni^{2+} was determined by measuring the amounts of Ni^{2+} in the inside and outside vesicle using an atomic absorption flame emission spectrophotometer (Model AA-6701F; Japan).

Protein Determination

Protein concentrations were determined by a bicinchonic acid assay kit [29] using bovine serum albumin as a standard.

RESULTS

Effect of ATPase Inhibitors on Bacterial Growth

To elucidate the anti-*H. pylori* mechanism of ATPase inhibitors, a number of bacterial strains were tested for their susceptibilities to these compounds and compared with that found in *H. pylori*. Using agar media seeded with fresh cells of an average concentration of 5×10^6 cells/ml, the disk zones of growth inhibition due to the presence of each compound were measured and plotted as a function of the logarithmic concentrations of the corresponding compounds. The data obtained are shown in Fig. 1. The azide MICs toward bacterial strains other than *H. pylori* cells were estimated by extrapolating the slopes until reaching a position of 8 mm, ranging 0.7±0.5 μmole/disc. In contrast, *H. pylori* was markedly resistant to azide, exceeding by 10-fold the average MIC found in other bacteria, resulting to give a bacteriostatic effect. Conversely, the effect of azide against the other strains was all but bactericidal. In addition, within 1 μmole/disk, neither OMP nor OAS exhibited antibacterial properties toward strains other than *H. pylori* (data not shown). From these observations, *H. pylori* proton pumps (F-type and P-type ATPases) are thought to possess unique specificities toward ATPase inhibitors.

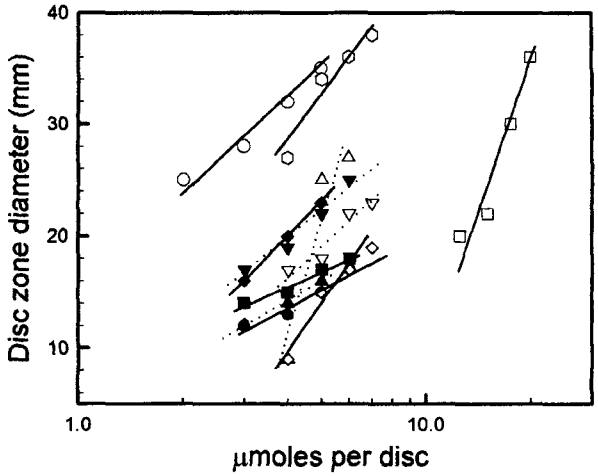

Fig. 1. Determination of antibacterial properties of ATPase inhibitors.

Using petri plates, seeded agar media (5×10^6 cells/ml) containing culture media for individual strains were prepared (see 'Materials and Methods' for compositions). Solutions of the compounds were added to paper disks (8-mm diameter; Toyo Roshi Kaisha, Ltd., Japan) and the disks were transferred to the plates. After incubation under individual culture conditions, the diameters of the growth inhibition zones were measured, respectively. Symbols used: (—○/●—), for *K. aeroginosa*; (—◌—), for *B. subtilis*; (·· △ /▲··), for *E. coli*; (— ◇ /◆—), for *P. mirabilis*; (·· ▽ /▼··), for *S. typhymurium*; (— □/■ —), for *H. pylori* with azide (open) or vanadate (closed), respectively. Note that in some cases, 'static' yet not 'cidal' zones were observed (e.g., *H. pylori*, *P. mirabilis*, or *S. typhymurium* with azide and *K. aeroginosa* with vanadate).

Anti-*H. pylori* Properties of ATPase Inhibitors

The ATPase inhibitors mentioned above were tested with *H. pylori* strains. As predicted, for exposure within the range of about 10 μmoles of azide/disk, all strains were shown to be bacteriostatic with relatively close MIC values. In contrast, their sensitivities to vanadate were considerably different from each other (Table 1). Furthermore, with this compound, certain strains such as *H. pylori* G880331 and G880221 formed large bacteriostatic zones exterior to the

Table 1. Anti-*H. pylori* spectra of ATPase inhibitors.

H. pylori Strain	MIC (μmoles/disc)		
	Azide	Vanadate	OMP
49503[a]	6.6	0.90	0.19
P3[b]	2.9	0.64	0.12
43504[a]	6.8	ND	0.18
331-1[c]	ND	0.16 (0.1)[d]	0.08
G88017-1[c]	2.7	0.58	0.07
G88022-1[c]	ND	0.86 (0.48)[d]	0.10 (0.07)[d]
O4[b]	4.7	0.82	0.09
43526[a]	ND	0.34	ND
51-1[c]	ND	ND	ND

[a]ATCC type strains.
[b]Authentic from VAMC, Los Angeles (U.S.A.) [29].
[c]Authentic from Medical College, Kyung Sang University (Korea).
[d]Parenthesized values indicate bacteriostatic zone diameter.
ND; not detectable under the concentrations examined.

bactericidal zones (data not shown). According to the slopes achieved by the above method, those formed with OMP and OAS were similar to those observed with *N,N'*-dicyclohexylcarbodiimide (DCCD) or *N*-ethylmaleimide (NEM). It should be noted that the inhibitory mode of vanadate on *H. pylori* growth resulted in a slope with a gentle gradient, indicating that its mechanism of action inhibiting *H. pylori* growth might be different from those found in the above compounds (Fig. 2). Figure 2 depicts the antibacterial nature of bafilomycin A_1. This compound is generally known to inhibit only V-type ATPase in living systems [8], however, it is found to be a powerful antibiotic toward *H. pylori*. The existence of V-type ATPase in *H. pylori* has not yet been reported. Hence, the anti-*H. pylori* mechanism of bafilomycin A_1 is not evident.

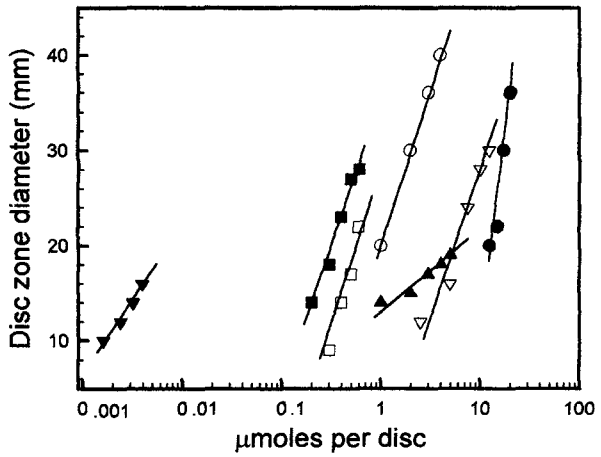

Fig. 2. Effect of ATPase inhibitors on *H. pylori* growth.
Detailed conditions for the disc zone test were the same as in Fig. 1. Symbols used: (□) OMP, (■) OAS, (●) azide, (○) DCCD, (▼) bafilomycin A_1, (▽) NEM (▲) vanadate.

Synergy between Vanadate and OMP/OAS

Since only vanadate seems to inhibit at the catalytic site of P-type ATPase, its possible synergistic inhibition of *H. pylori* growth with others such as OMP was anticipated. By using the disk zone test, we ascertained that the growth inhibition by vanadate, especially at low concentrations (less than 0.1 μmoles/disk or less) where no visible zones could be observed, was markedly enhanced (over 10-fold at about a 10-mm diameter disk zone) with the aid of 0.1 μmoles of OMP/disk which did not show any visible zone by itself (Fig. 3). Note that, under such conditions, the increase in the zone diameter (bactericidal) with increasing the vanadate concentration was negligible. Azide also synergistically inhibited the *H. pylori* growth when supplemented with 0.1 μmoles of OMP or OAS/disk. From the shift-patterns of the slopes with synergisms, it is suggested that OMP and OAS may potentiate a certain target for attenuation along with azide or vanadate, although these are normally known as inhibitors for F-type and P-type ATPases, respectively [11].

To clarify the significance of these synergisms, membranes were isolated and the effect of ATPase inhibitors on P-type ATPase was studied. As presummed it was found that in *H. pylori* membranes the azide-sensitive fraction was far less compared to the vanadate-sensitive one (Fig. 4). Interestingly, when repressing the F-type ATPase with azide, the remaining ATPase activity was proportionally reduced by increasing the vanadate concentration. With an additional presence of OAS (0.02–0.2 mM), however, the effect of vanadate at low concentrations (0.1 mM or less) reducing enzyme activity was remarkable, exhibiting sigmoidal inactivation. This phenomenon was thought to be due to the synergistic

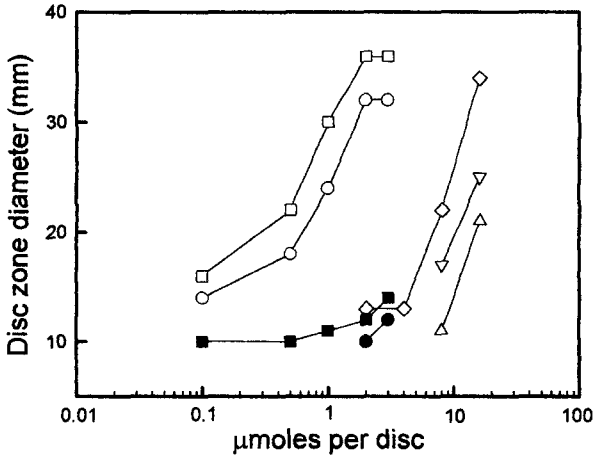

Fig. 3. Inhibitory synergy of ATPase inhibitors on *H. pylori* growth.
Symbols used: (○/●) vanadate only, (□/■) vanadate plus 0.1 mM OMP, (△) azide only, (▽) azide plus 0.1 mM OMP, (◇) azide plus 0.1 mM OAS. Open and closed symbols indicate bacteriostatic and bactericied zones, respectively.

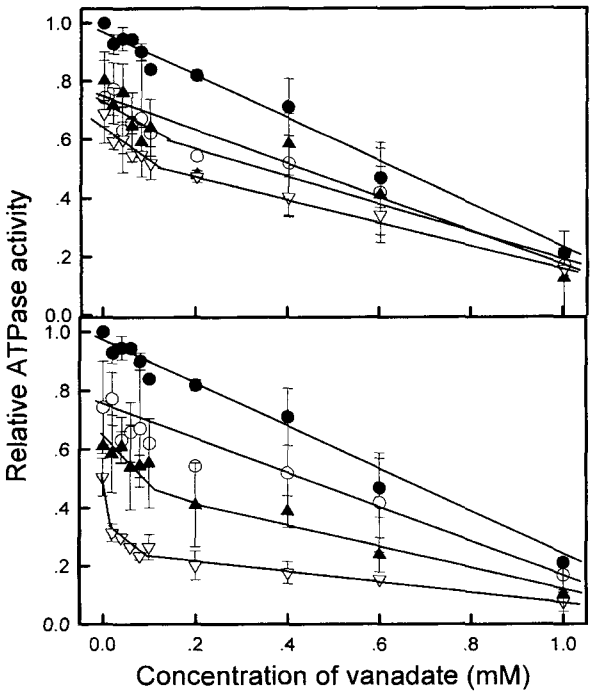

Fig. 4. Synergistic effect of ATPase inhibitors on *H. pylori* membrane P-type ATPase activity.

Membrane vesicles (40 μg proteins) were added to solutions containing various concentrations of vanadate and fixed concentrations of other ATPase inhibitors, and the resulting mixtures were incubated for 10 min at 37°C before the addition of ATP. (●) vanadate only, (○) with 0.2 mM azide, (▲) with 0.2 mM azide and 0.02 mM OMP (A) or OAS (B), (▽) 0.2 mM azide and 0.2 mM OMP (A) or OAS (B).

action of vanadate (≤0.2 mM) and OAS in inhibiting the P-type ATPase activity. Such a sigmoidal inhibition but with a gentle gradient was also observed with OMP, indicating its spontaneous conversion to OAS under the experimental conditions. Taking this inhibitory mode into account for cell death (refer data in Fig. 3), these seem to be closely related each other.

Role of *H. pylori* P-type ATPase Activity on Metal Ion Transport

Using the everted membrane vesicles saturated with $NiCl_2$, the export rate of nickel ion in the presence or absence of ATP or ATPase inhibitors was determined. As shown in Table 2, with the aid of ATP and NH_4Cl, the Ni^{2+} transport rate across *H. pylori* membrane was increased up to 50% from the basal rate. In addition, the ATP/NH_4^+-dependent transport of Ni^{2+} was completely depressed by the presence of vanadate, strongly suggesting that Ni^{2+} transport in *H. pylori* may be controlled in part by NH_4^+-motive P-type ATPase. In separate tests, it was found that monovalent cations such as Na^+ or K^+ acted as positive effectors for P-type ATPase activity (data not shown). However, whether or not these cations can behave like NH_4^+ is still under investigation.

Table 2. Factors affecting Ni^{2+}-export in the everted membrane vesicle from *H. pylori*.

Addition	Ni^{2+} (nM)	
	Intravesicular	Extravesicular
None	0.32±0.04	0.28±0.04
ATP	0.32±0.04	0.36±0.03
NH_4Cl	0.34±0.03	0.32±0.03
ATP, NH_4Cl	0.31±0.03	0.41±0.04
ATP, NH_4Cl, vanadate	0.51±0.05	0.23±0.04

The everted membrane vesicles saturated with Ni^{2+} were incubated either with or without additives for 30 min at 37°C. After incubation, the vesicle suspensions were filtered using a polysulfone-membrane tube by centrifugation (4°C; 9,000 ×g for 30 min). The amounts of Ni^{2+} in the vesicles and filtrates were determined by the method as described in 'Materials and Methods'. Concentrations of factors used: ATP, 1 mM; NH_4Cl, 10 mM; sodium ortho-vanadate, 0.1 mM.

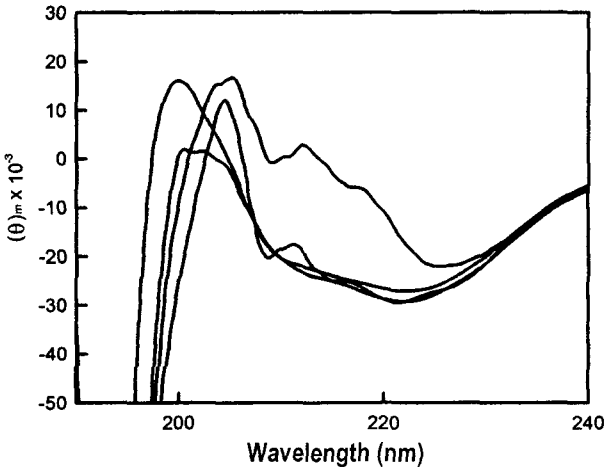

Fig. 5. Effect of OAS on circular dichroism spectra of *H. pylori* membranes.

Curves from left to right (at far UV regions): Control, 0.01 mM OAS, 1 mM OAS, and 0.1 mM OAS.

CD Spectra of Membrane Proteins of *H. pylori*

H. pylori membranes were treated with OAS at various concentrations and the CD spectra were read. The resulting data are presented in Fig. 5. As shown in this figure, the treatment of membrane vesicles with 0.1 mM OAS, near the MIC for *H. pylori* growth, caused a considerable shift of absorption peak (198 nm to 205 nm). By increasing the OAS concentration, the optical densities of vesicles were markedly increased within the range of 210 nm to 230 nm, indicating that the secondary helical structure of the membrane proteins appeared to be disrupted. Accordingly, it was thought that both the inactivation of membrane ATPase and cell death would have occurred before the helical structure was disrupted. In fact, a reducing agent such as dithiothreitol could be applied to recover its ATP hydrolytic activity, when under a mild treatment with OAS (data not shown). It seems that a certain SH-group located

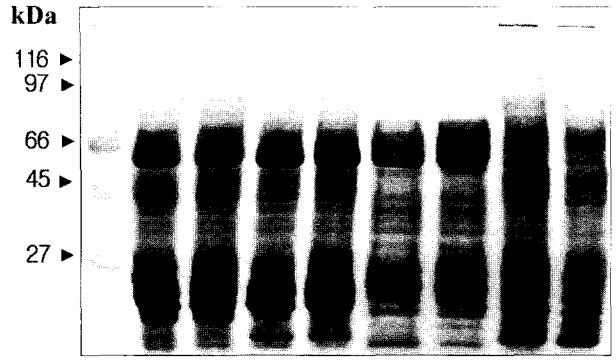

Fig. 6. Electrophoretic assessment of protein phenotypes of *H. pylori* after treatment with OMP.
SDS-PAGE was performed according to the method of Laemmli (1% SDS; 10% polyacrylamide gel) [18]. Lanes from left to right; molecular weight marker proteins, extracellular proteins, extracellular proteins with 0.1 mM OMP, periplasmic proteins, periplasmic proteins with 0.1 mM OMP, cytosolic proteins, cytosolic proteins with 0.1 mM OMP, membrane proteins, and membrane proteins with 0.1 mM OMP.

in the cytoplasmic domain is involved in the enzyme catalysis.

Effect of OMP on the Protein Expression of *H. pylori* Cells

Previously, it was noted that both OMP and OAS at a neutral pH were readily taken up by *H. pylori* cells and bound to the catalytic domain of P-type ATPase (our observation). To understand whether or not OMP could confer a lethal change to the cell's other physiology as well, an experiment accessing OMP's effect on cellular protein expression was performed. After treatment with OMP, cellular fractionation was carried out, followed by SDS-PAGE. The result is presented in Fig. 6. Unexpectedly, the two bands in the cytoplasmic fraction, positioned respectively at molecular weights of ca. 62 and 56 kDa, were evidently intensified. A change in the low molecular weight region (15 kDa) was also observed. From this finding, it was indicated that OMP or OAS could be taken up by *H. pylori* cells, altering the cell's protein phenotype in addition to forming disulfides (Ki *et al.* 1999. *J. Microbiol. Biotechnol.* in press). Elucidating whether or not the change in the cytoplasmic proteins by OMP does pertain to *H. pylori* death was beyond the scope of this study.

DISCUSSION

The interactions between *H. pylori*, gastric acid, and proton pump inhibitors (PPIs) that suppress acid secretion remain to be a topic of current interest. Suppressing acid production has been known to affect bacterial colonization, but it does not eradicate *H. pylori* even if the situation lasted for years [17]. With omeprazole (OMP), however, the *H. pylori* eradication rate of antibiotics (amoxicillin plus clarithromycin) was reported to be increased by 3.6-fold, suggesting a synergistic effect of OMP with antibiotics [19]. Additionally, OMP reduces the risk of both primary and secondary resistance. According to the pharmaceutical studies, OMP is known to be readily absorbed via the mucous layer, even reaching the canaliculi of parietal cells whereby it is concentrated by converting to a permanent cationic nature in acid [12]. This in turn, reflects that this compound has easy access to *H. pylori* that are deeply infected. Such an agent is considered useful in developing rational chemotherapy for patients infected with *H. pylori*. Elucidating a mechanism of how PPIs like OMP affect *H. pylori* cells *in vivo* is a current matter of concern. Manifesting their crucial target in *H. pylori* cells may account for the development of a novel agent so that *H. pylori in vivo* can effectively be eradicated.

Recently, it was found that the P-type ATPase activity present in *H. pylori* membranes was essential for the peptide transport in *H. pylori* (M. R. Ki *et al.* 1998. *Abstr. Fall Meet. Kor. Soc. Biochem.* p. 30). To demonstrate the relationship between this activity and cell growth, this paper examined two different kinds of enzyme inhibitors, i.e., vanadate and OMP (OAS), known to differ in their inhibitory mechanisms. By employing a disk zone analysis, their synergic action in cell growth inhibition was identified, particularly when the concentration of one of these compounds was far lower than its apparent MIC (Fig. 3). Similarly, the P-type ATPase activity was severely attenuated under such conditions (Fig. 4). From these observations, it was strongly suggested that inactivation of this enzyme could be an immediate cause for *H. pylori* death. It would appear, therefore, that for active transport by cells, P-type ATPase is specifically needed in *H. pylori* (Ki *et al.* 1999. J. Microbiol. Biotechnol., in press). Considering that the ATPase activity in the *H. pylori* membranes appeared to be exclusively composed of vanadate-sensitive enzymes [33], the above theory is quite feasible.

H. pylori cells possess large amounts of ATP phosphatases which appear to be preferably vanadate-sensitive (S. K. Yun *et al.* 1998. *Abstr. Annu. Meet. Kor. Soc. Microbiol.* p. 20). *H. pylori* membrane vesicles are also aggregative, capable of holding unwanted ATPases [33]. Therefore, permeabilized cells or membranes of just French-pressed, membrane-bound P-type ATPase could easily be contaminated by soluble or cell surface ATPases. Accordingly, evaluating the physiological role of this enzyme in *H. pylori* is difficult. In this paper, this problem was circumvented by preparing cytoplasmic membranes from *H. pylori* spheroplasts and by diluting the membrane vesicles. We used these vesicles for the transport assay. In Table 2, nickel ion was released without such additives as ATP or NH_4Cl, and the rate reached to about 70% of that

found with the additives. This suggests that the cation motive P-type ATPase may be partly involved in nickel ion transport. The NixA (a high-affinity nickel transport protein) of the *H. pylori* membrane is also known as a transport factor [2]. In this regard, the mechanism of Ni^{2+} transport in *H. pylori* is complex. However, since this metal ion is essential for intracellular maturation of urease, an investigation of its transport would seem to be important.

In order to function on a cytoplasmic membrane, P-type ATPase requires a continuous supply of ATP. However, since the F-type ATPase activity was determined to be far less than the P-type ATPase activity, the ATP-generating machinery (utilizing D-glucose or amino acids as substrate) other than the F-type ATPase must be properly operated for cell metabolism. A number of P-type ATPase genes are thought to be expressed in *H. pylori* [5, 22]. However, the nature of the P-type ATPases in this organism is still uncertain. Nonetheless, it seems highly probable that this enzyme activity is an essential factor of *H. pylori* physiology for the following reasons: Firstly, for the uptake of essential nutrients (e.g., amino acids or peptides) or minerals, *H. pylori* cells require the active P-type ATPase. Secondly, the synergistic inhibition of *H. pylori* growth by ATPase inhibitors seems to be closely associated with that found in P-type ATPase activity. Finally, *H. pylori* cells produce huge amounts of ATP phosphatases of which only the P-type ATPase activity of *H. pylori* membrane is retained when the cells encounter acid (pH 3.2; border pH for *H. pylori* survival) [33]. Taking into account this enzyme to be indispensible to *H. pylori* physiology, development of novel agents that are physicochemically similar to OMP will provide successful eradication of *H. pylori*, which is deeply infected.

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