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Effect of Omeprazole on Membrane P-Type ATPase and Peptide Transport in *Helicobacter pylori*

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Abstract We investigated the growth-inhibitory mechanism of *Helicobacter pylori* by omeprazole (OMP) and its activated sulfenamide (OAS). Using dithiothreitol (DTT) and 5,5'-dithio-bis[2-nitrobenzoic acid] (DTNB; Ellman's reagent), we first determined the relationship between the binding capacity of these compounds to *H. pylori* membrane and its significance to membrane P-type ATPase activity. After incubation of the intact *H. pylori* cells with either OMP or OAS, the residual quantity of free SH-groups on the cell membrane was measured, and, the resulting values were plotted as a function of time. From this experiment, we found that there was a considerable difference in the membrane-binding rates between OMP and OAS. At neutral pH, the disulfide bond formation on *H. pylori* membrane was completed within 2 min of incubation of the intact cells with OAS. By OMP, however, it was gradually formed, exceeding 10 min of incubation for completion, whereby, the extent of P-type ATPase inhibition appeared to be proportional to the disulfide forming rate. From this data, it was suggested that the disulfide formation might directly affect enzyme activity. Since OMP *per se* cannot yield a disulfide bond with cysteine, it is predicted that the enzyme inactivation must be caused by the OAS form. Accordingly, we postulated that, under the neutral pH, OMP could be converted to OAS in the course of transport. By extrapolating the inhibitory slopes, we could evaluate K_i values, relating to their minimal inhibitory concentrations (MICs) for *H. pylori* growth. In these MIC ranges, *H. pylori* uptake or vesicular export of nutrients such as peptides were totally prohibited, but their effect in *Escherichia coli* were negligible. From these observations, we strongly suggest that the P-type ATPase activity is essential for the survival of *H. pylori* cells in particular.

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

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Etiologically, gastric infection by *Helicobacter pylori* is of great significance to human health although the majority of *H. pylori*-infected persons remain asymptomatic [8, 9, 10]. As a major causative agent, this bacterium is currently recognized as being associated with acute, chronic, and recurrent symptoms of gastritis or peptic ulcer [31]. Once *H. pylori* causes such diseases, the symptoms become chronical and may potentiate gastric carcinoma [28, 39]. Several pathogenic factors implicated in this intractable disease, i.e., urease, VacA (cytotoxin), and membranous lipid components, etc., have been extensively studied [1, 11, 29, 30]. Currently, eradication of *H. pylori* is the major issue in preventing such a serious complication of this gastric disease. Unfortunately, despite the numerous reports in relation to eradicating *H. pylori*, successful data have not yet been obtained *in vivo* [14, 19, 35]. The viability of *H. pylori* cells from their random exposure to strong acid (pH 1-2) or antibiotics may be attributed to its tendency of deep colonization within the viscous mucus layer of stomach or adhesion to gastric parietal cells [9, 36].

So far, none of the bacterial species other than *H. pylori* are known to persist in the human stomach as an ecological niche. The mechanism and significance of gastric growth of this organism is uncertain. To survive, *H. pylori* must simultaneously possess at least two mechanisms; one for deep colonization and the other for acid tolerance. In the latter case, the possible involvement of proton pumps has been suggested [23]. Previously, we reported on the significance of differential exhibition of proton pumps on *H. pylori* membranes, where a P-type ATPase pool was predominant [40, 41]. Other bacterial membranes (e.g., *Escherichia coli*, *Enterococcus faecalis*, or *Salmonella typhimurium*, etc.), however, are constituted exclusively of F-type ATPase with extra P-type ATPase [12, 13]. These proton pumps are involved either in ATP synthesis (F-type) or in the regulation of pH and cation concentrations across cell membranes (P-type), but the role of neither P-type nor F-type ATPases in *H. pylori* is yet fully understood [2, 24, 26].

Gastric proton pump inhibitors (PPIs), i.e., substituted benzimidazole derivatives (OMP, etc.) have been used to cure gastric disease. After recognizing the nature of *H. pylori*, however, chemotherapy for patients infected with this bacterium often includes extra antibiotics. Unfortunately, most antibiotics commonly used are not potent enough to eradicate *H. pylori* for several reasons; lack of reaching to deeply infected cells, deficiency in the drug action towards dormant cells, or instability of the drugs under acid environment, etc. In contrast, PPIs such as OMP can readily penetrate the gastric mucus barrier, and concentrate even in the secretory canaliculus. OMP has now been clinically proven to be safe [4]. Recently, OMP was found to inhibit *H. pylori* growth *in vitro*. Based on the pharmaceutical potential, a compound like OMP seemed the ideal agent to eradicate *H. pylori*. Understanding the anti-*H. pylori* mechanism of OMP is therefore a prerequisite. Although studies have been made to clarify the crucial target of this compound in *H. pylori* cells, none of the reports have successfully elucidated the mechanism of action of OMP [4, 22, 23, 34]. Because of the cationic nature, its sulfenamide (OAS) is known to be impermeable to plasma membrane of gastric parietal cells [6]. Regarding its transport in *H. pylori*, no data have been reported. By using the intact *H. pylori* cells, however, we recently found that OMP as well as OAS could easily be taken up by *H. pylori* cells, forming a disulfide at the cytoplasmic domain of P-type ATPase.

In this paper, we describe the inhibitory mechanism of OMP and OAS toward P-type ATPase activity and show its consequence on peptide transport in *H. pylori*. For comparison, *E. coli* cells were also examined.

Materials and Methods

Microorganisms and Cell Growth

H. pylori ATCC 49503 was obtained from the American Type Culture Collection (Rockville, U.S.A.). *E. coli* HB101 was kindly provided from Lipid laboratory, VAMC, Los Angeles, U.S.A. *Bacillus megaterium*, *Staphylococcus aureus*, *Klebsiella aerogenes*, and *Proteus mirabilis* were authentic strains from Korean Type Culture Collection, KRIBB (Korea) [16]. Frozen cells of *H. pylori* (-80°C) were thawed, inoculated into brain heart infusion medium (Difco, Detroit, U.S.A.) containing 5% bovine serum and grown at 37°C under controlled air composition (5% O_2, 10% CO_2, and 85% N_2). After 2 days of cultivation, cells were harvested either by scraping out colonies from agar plates or by centrifuging the culture broth at 9,000 ×g for 10 min [39]. Other bacterial strains were cultivated by reciprocal shaking (100 rpm) at 37°C using LB liquid medium.

P-Type ATPase Assay

To measure P-type ATPase activity in *H. pylori* cells, everted membrane vesicles were prepared as follows: Cells were disrupted by ultrasonic treatment and ultracentrifuged (190,000×g, 60 min) as described previously [41]. Following the preparation of vesicle suspension (10- 100 μg proteins/ml; 20 mM Tris-HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and 0.25 M sucrose), ATP hydrolysis was then initiated by the addition of 2 mM ATP. In a typical assay, the enzyme reaction in a final volume of 1 ml was carried out for 30 min at 37°C. After incubation, the reaction mixture was transferred to an ice bath. The liberated inorganic phosphate was quantified according to the method of Yoda and Hokins [38]. Briefly, to the reaction mixture was added 1 ml of pre-chilled 12% perchloric acid containing 3.6% ammonium molybdate and extracted molybdophosphate complex by 3 ml of *n*-butylacetate. The ATPase activity was determined by measuring its quantity at 320 nm (UV 2101 spectrophotometer; Shimadzu).

Active Transport of Peptides

Bacterial transport of peptide was determined using the intact cell suspensions (A_{660}=0.5; 50 mM Tris-HCl, pH 7.4). The intact cells were pretreated with ATPase inhibitors (i.e., 0.1 mM azide, vanadate, OMP, or OAS) for 10 min at 37°C before the addition of 0.1 mM L-phenylalanyl-L-3-thia-phenylalanine (PSP) [18] and DTNB. If necessary, ATPase inhibitors were added at the beginning of transport. The reaction mixture with a final volume of 1 ml was placed in 1-ml light-path cuvette and incubated at room temperature for the transport assay. The released thiophenol upon PSP hydrolysis by intracellular peptidase was reacted with DTNB, yielding an anionic yellow adduct. Its absorbance was recorded spectrophotometrically at 412 nm [15].

The vesicular export of L-alanyl-L-alanine was studied as follows; to the everted membrane vesicle suspension (1 mg protein/ml; 20 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose), the above dipeptide at 50 mM (find conc.) was added and incubated overnight on ice with gentle shaking. After incubation, the suspension was ultracentrifuged (190,000×g, 60 min). The sedimented membrane pellet was carefully washed by resuspending into the above buffer. The washed vesicle suspension (30 μg protein/0.1 ml) was placed into test tubes, and pretreated at 37°C for 5 min with 0.1 mM of each ATPase inhibitor before the addition of ATP. After incubation of the reaction mixture (37°C, 30 min), 5% sodium borate buffer (containing 1 mM 2',4',6'-trinitrobezene sulfonate [pycryl sulfonate; TNBS]) was added to give 1 ml. After standing for 30 min at room temperature, the amount of yellow adduct was determined by measuring its absorbance at 420 nm [7].

Determination of Disulfide Formation by OMP or OAS

Time course treatment of the intact cells with 0.1 mM OMP or OAS was performed. Following the disruption of the cells, membranes were isolated by ultracentrifugation

as described before. Membranes were then solubilized by 1% SDS, and the amount of disulfides was determined by measuring free SH groups with DTNB before or after treatment with 1% DTT.

Chemicals

PSP was prepared in our laboratory. In short, *N*-carbobenzoxy-L-phenylalanylamide benzyl glyoxylate was reacted to give the *N*-carbobenzoxy-L-phenylalanyl-D,L-2-hydroxyglycine benzyl ester. By successive acetylation at the 2-hydroxyl moiety, an acetoxy synthon was made. After substitution of the acetoxy group by thiophenol, stereoisomers were separated by the method of crystallization. L,L-PSP was obtained by deblocking with HBr in AcOH [17, 18]. ^{1}H NMR (5% DCl/D_2O) δ3.2 (d, 2H), 4.4 (t, 1H), 5.6 (s, 1H), 7.1-7.7 (m, 10H). OMP was kindly provided from Jongundang Pharmaceutical Co., Korea. OAS was prepared by treatment of OMP with acidic methanol (pH 3.0) [3].

Protein Assay

Protein concentration was assayed with a BCA assay kit (Pierce Co.) [37] according to the modified Lowry method.

RESULTS

Antibacterial Activities of OMP and OAS

To evaluate the antibiotic properties of OMP and OAS, we performed a disc zone test for growth inhibitions toward a number of bacterial strains including *H. pylori*. Under the experimental conditions, these compounds exhibited relatively potent anti-*H. pylori* property compared to the other bacteria tested. All bacteria except *H. pylori* were resistant to OMP but OAS could act as an antibiotic towards certain bacteria (Table 1). Assuming that the antibiotic property of OAS would exclusively be the consequence of inactivation of a certain lethal enzyme by forming the disulfide bridge with cysteine residues in the enzyme, the antibacterial potential of OMP might be due to its convertibility as OAS during bacterial transport. In fact, OMP *per se* cannot yield a disulfide bridge with cysteine. Thus, OMP must be converted to OAS prior to reacting with the SH-group (data not shown). The growth-inhibitory disc zone patterns caused by OAS appeared to be different in each case, suggesting that the individual targets and their role in cell physiology would be different from each other.

Table 1. The effect of OMP and OAS on bacterial growth.

Strains	Diameter of antibacterial zone (mm)							
	OMP (mM)				OAS (mM)			
	0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4
H. pylori	8	12	14	16	14	19	21	24
E. coli	-	-	-	-	12	14	16	18
S. aureus	-	-	-	-	(12)	(14)	(17)	(18)
B. subtilis	-	-	-	-	10	12	13	15
P. mirabilis	-	-	-	-	-	-	-	-
K. aerogenes	-	-	-	-	-	-	-	-

Solutions of the compounds were added to filter paper disks (8-mm diameter) and the disks were transferred to the agar plates seeded with bacterial cells (1×10^7 cells/ml; refer 'Materials and Methods' for medium compositions). After incubation at 37°C until cell colonies were visualized, the diameters of the zones of inhibition were measured. MIC was estimated by extrapolating the slope obtained from a plot of disc zone diameter as a function of logarithmic concentration of drug applied. Parentheses indicate bacteriostatic zone. -, Not visibly detectable.

Effect of OMP on the Membrane ATPase Activities from *H. pylori* and *E. coli*

Using everted membrane vesicles from *H. pylori*, the effect of OMP concentration on ATPase activities was

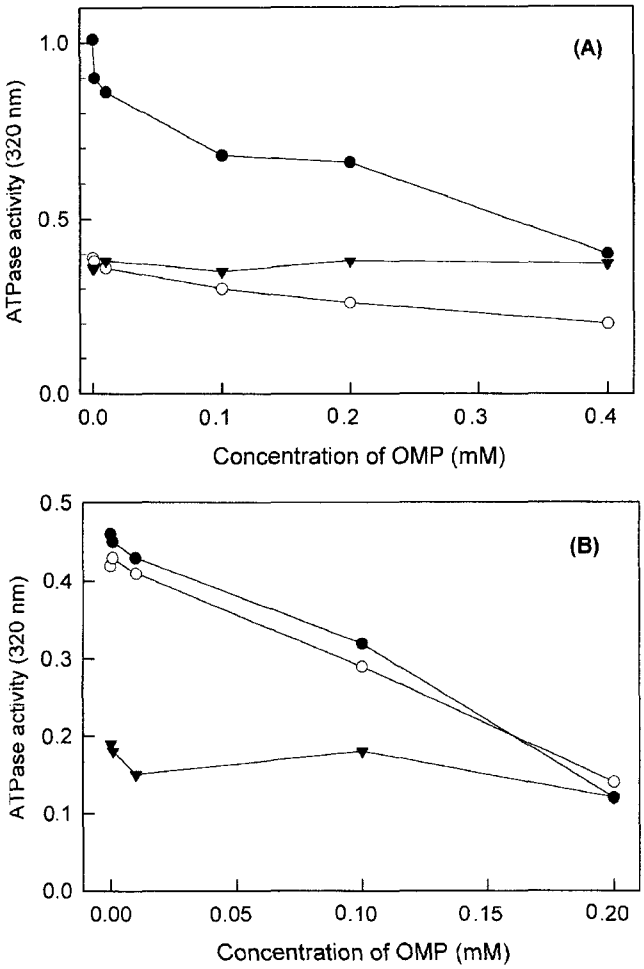

Fig. 1. Effect of OMP concentration on membrane ATPase activities of *E. coli* and *H. pylori*.

Membrane vesicles (100 μg proteins/ml), preincubated (37°C, 5 min) with or without ATPase inhibitors, were treated again with OMP (37°C, 10 min) before the addition of ATP. (A), *E. coli*. (B), *H. pylori*. Symbols used: (●), OMP only; (○), OMP with 1 mM azide; (▼), OMP with 0.4 mM vanadate.

examined. *E. coli* was employed as a control. The vanadate-sensitive enzyme pools (P-type ATPase) were considerably inhibited while those sensitive to azide (F-type ATPase) were rather tolerant in both strains (Fig. 1). Notably, the whole enzyme activities were not proportionally decreased by increasing the OMP concentrations. This phenomenon was more apparent in the case of *H. pylori*, where the hypothetically computed gap between specific and non-specific inhibition of vanadate-sensitive ATPase activity was far greater than that found in the case of *E. coli*. We thought that this gradually increasing severity in the ATPase inhibition might be due to the spontaneous alterability of OMP to OAS. Therefore, the potential of OMP's structural shift to OAS, even under mild conditions (e.g., neutral pH), should not be ignored in *H. pylori*. It was noteworthy that the half point of the gap was assessed to be at 0.1 mM OMP, found to be close to the MIC value (refer to the legend in Table 1 for evaluation) for *H. pylori* growth.

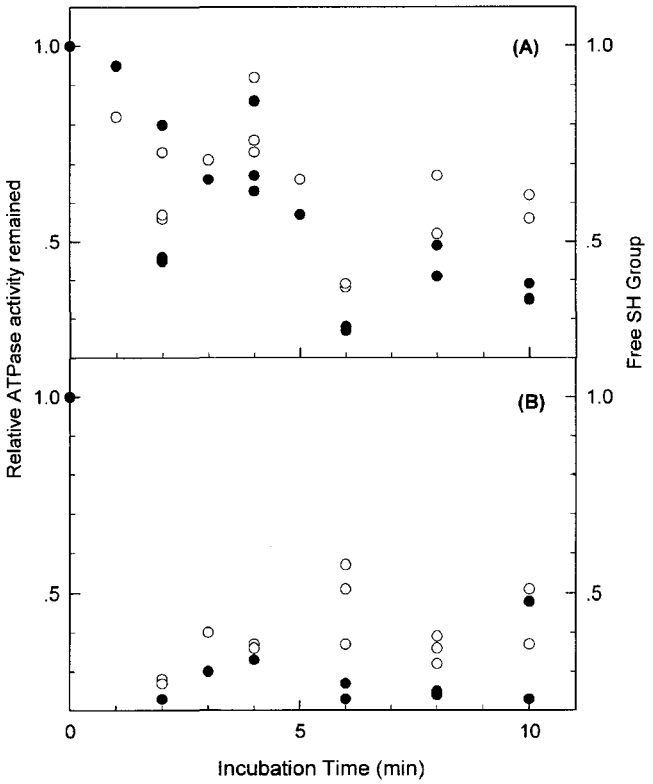

Fig. 2. Relationship between disulfide formation and P-type ATPase inhibition by OMP and OAS in cell membrane of *H. pylori*.

H. pylori cells (A_{660}=0.5) were incubated in the presence of 0.1 mM OMP (A) or OAS (B). Portions of cell suspension were taken at 1-min intervals and frozen immediately. The resulting cells were then thawed, disrupted, and ultracentrifuged to isolate cell membranes as described in 'Materials and Methods'. The relative amounts of remained free SH-group (○) or ATPase activity (●) from controls (1.0 at 0 min) were plotted against incubation time.

Determination of Disulfide Formation by OMP and OAS: Its Relationship to P-Type ATPase Activity

Aiming to gain further evidence concerning the participation of P-type ATPase activity on *H. pylori* growth, an experiment employing its intact cells was carried out as follows: After treatment of cells with OMP or OAS, relevant membrane vesicles were prepared. The amounts of cysteine residues occupied by OMP or OAS were plotted as a function of time. The time-dependent decrease in the P-type ATPase activity was also assessed (Fig. 2). Obviously, most disulfides were formed (ca. 60% of whole cysteine residues) within 2 min of exposure of the cells to 0.1 mM OAS, accompanied with complete inactivation of the P-type enzyme activity. Until this time, however, both the level of disulfides or enzyme inactivation did not exceed 30% by OMP. It was interesting that the extent of enzyme

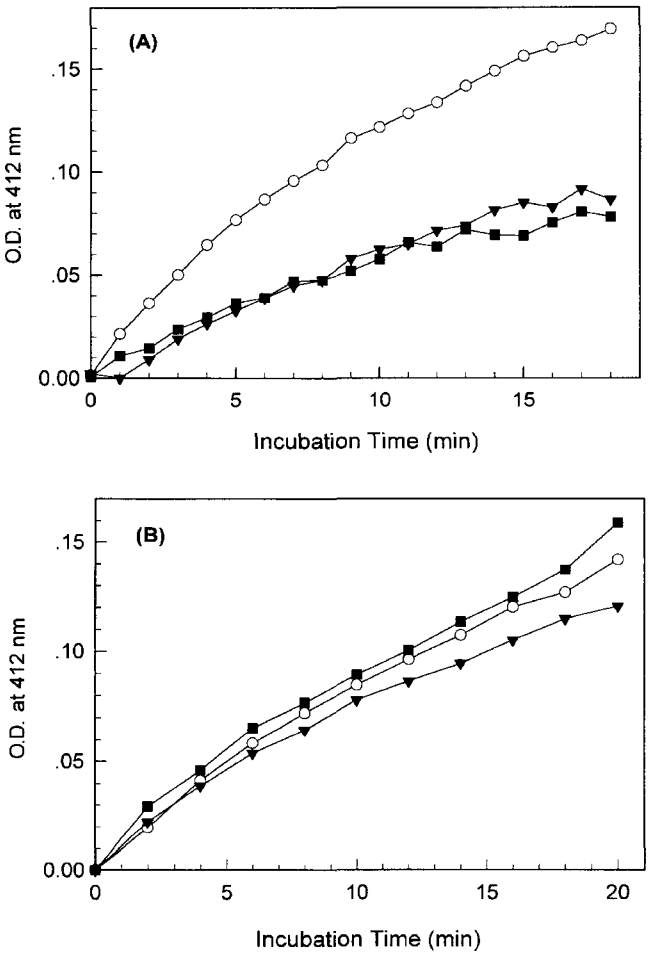

Fig. 3. Effect of ATPase inhibitors on the bacterial transport of PSP.

Intact *H. pylori* (A) or *E. coli* (B) cell suspension (A_{660}=0.5, 0.1 mM DTNB in 50 mM Tris-HCl buffer, pH 7.4), incubated for 10 min at 37°C with or without ATPase inhibitors, was placed in a 1-ml light-path cuvette. The transport reaction was then initiated by adding PSP (0.1 mM) to give a final volume of 1 ml. The amount of yellow adduct was determined at 412 nm by a spectrophotometer (refer to text for details). Symbols used: (○), no inhibitors; (▼), 0.1 mM azide; (■), 0.1 mM vanadate.

inhibition was precisely related to the amounts of disulfide formed. Considering the ca. 2-fold difference between the two compounds in MIC, the enzyme inhibition via disulfide formation seemed to directly affect the cell growth. This, in turn, illustrates that both compounds can be taken up effectively by *H. pylori* cells and form disulfide bridges at the catalytic domain of P-type ATPases, resided in the cytoplasm. Because of its cationic nature, it is generally accepted that OAS cannot penetrate into the cytoplasm of gastric parietal cells [6]. In this regard, the cytoplasmic membrane of *H. pylori* is thought to be vulnerable.

Effect of ATPase Inhibitors on Cellular Import of Peptide in *H. pylori*

Since *H. pylori* is a typical parasite, various nutrients must be supplied for its survival. Their active transport was thought to be possible mainly by virtue of the function of P-type ATPase pool. To verify the inhibitory relationship between P-type ATPase and cell growth, we examined the effect of ATPase inhibitors on peptide transport as an example in *H. pylori*. Using PSP, a dipeptide mimetic, we could observe that the peptide transport in *H. pylori* was markedly inhibited by both azide and vanadate (Fig. 3A). Their effect in *E. coli*, however, was rather contradictory to the above data (Fig. 3B). From these observations, we clarified that P-type ATPase activity in the cell membrane of *H. pylori* was necessary at least for the active uptake of solutes.

The effect of OMP or OAS on PSP transport was also studied. Data in Fig. 4B show that the transport of PSP in *E. coli* was not affected at all by the presence of vanadate or OMP, but was evidently inhibited by OAS. This result is thought to be due to the inactivation of F-type ATPase but not P-type ATPase, indicating that F-type rather than P-type ATPase is essential for the survival of *E. coli*. In contrast, the PSP transport in *H. pylori* was affected by either of vanadate, OMP, or OAS (Fig. 4A). More interestingly, their action (OMP and OAS) inhibiting PSP transport appeared to begin after 2 min of incubation. This data is in good agreement with the necessary time to inactivate P-type ATPase in the intact *H. pylori* cells by OMP or OAS (Fig. 2).

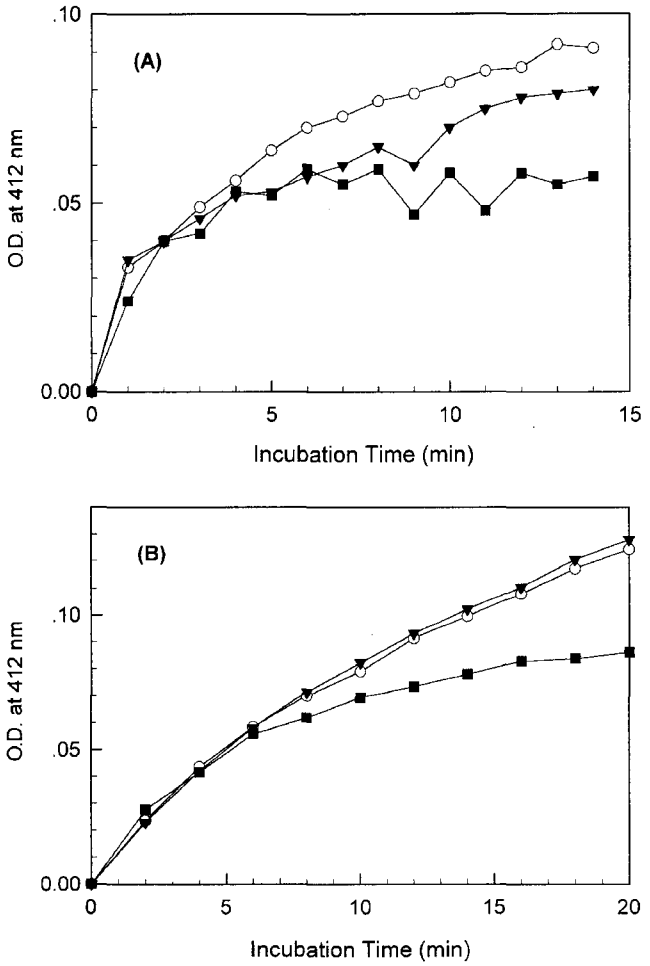

Fig. 4. Effect of OMP and OAS on the bacterial transport of PSP.

For general experimental conditions, see Fig. 3, except that in this experiment, cells were not pretreated with inhibitors. PSP was transported in the absence (○) or presence of 0.1 mM OMP (▼) or 0.1 mM OAS (■). Note that the effect of additives appeared after 4 to 5 min of PSP transport. (A) for *E. coli*; (B) for *H. pylori*.

Effect of ATPase Inhibitors on the Vesicular Export of Peptide in *H. pylori*

To obtain more direct evidence for the involvement of P-type ATPase activity on peptide transport in *H. pylori*, the effect of the above inhibitors on the vesicular export of L-alanyl-L-alanine was also examined, and the result is given in Table 2. The data evidently showed that the rate of the dipeptide export from membrane vesicles were dependent on ATP hydrolysis, exclusively by virtue of P-type ATPase. This phenomenon, denoting active transport of dipeptide at the expense of ATP, may reflect the data of azide effect on PSP transport in *H. pylori* (Fig. 3A).

Table 2. Effect of ATPase inhibitors on vesicular export of Ala-Ala.

Compound (mM)	Export rate (nmoles/mg/min)
Without ATP	1.53
With ATP	
none	2.86
+ azide	2.50
+ vanadate	1.77
+ OMP	1.35
+ OAS	0

Everted membrane vesicles (30 μg protein/ml, 50 mM Ala-Ala, 0.25 M sucrose in Tris-HCl buffer, pH 7.4) were pretreated for 5 min at 37°C with inhibitors before the addition of ATP. Extravesicular concentration of the dipeptide was determined after ultracentrifugation, as described in Materials and Methods.

Surprisingly, the extravesicular secretion of Ala-Ala was also monitored without ATP, and it was about one-half slower than with ATP. It was uncertain whether this event was caused by decreased rigidity of vesicular membranes upon its overnight incubation to become saturated with the dipeptide.

Discussion

OMP, one of the bezimidazolic PPIs, was recently found to contain anti-*H. pylori* activity *in vitro* [34]. Several reports have dealt with identifying the crucial target of OMP in this bacterium, but this remains unclear. Beil *et al.* [3] and Belli *et al.* [5] stressed on the anti-*H. pylori* mechanism of OMP, suggesting that the main cause for cell death by OMP would not be due to its inhibition of P-type ATPase activity. Their assay systems, however, were believed to be contaminated with excess amounts of soluble ATPases, abundant in both the cytoplasm and periplasm of *H. pylori* cells. From their studies, we could not ascertain whether they correctly determined membrane-bound ATPase activity, which was our concern in this paper.

OMP is generally accepted to inhibit a number of proteins including urease or proton pumps, etc. in *H. pylori*, which specifically binds cysteine residues in proteins via its structural shift as cationic sulfenamide (OAS) [33]. Previously, we reported about the significance of P-type ATPase distribution as a higher proportion than F-type ATPase of *H. pylori* membrane [41]. P-type ATPases are generally known to be associated with the function of metal ions [20, 25]. Since this enzyme is usually found in eukaryotic cell membranes rather than in bacteria, our finding as above was remarkable. We assumed therefore that this enzyme activity would pertain to the vital function of energizing the cell membranes or cation transports. In this regard, the OMP- or OAS-caused cell death was presumed to be the direct consequence of P-type ATPase inhibition. In this study, we found that the complete inactivation of enzyme activity could result only by masking the cysteine residues of the protein. From the peptide transport experiments employing both the intact cells and membrane vesicles, we also found that the ATP hydrolysis by P-type ATPase was a prerequisite for peptide transport in *H. pylori* but not in *E. coli*, because the inhibitory mode of dipeptide transport with the aid of OMP or OAS was similar to that found by vanadate. Figure 3A shows that PSP transport in *H. pylori* intact cells was markedly inhibited by azide. In contrast, azide exhibited no effect on the vesicular export of Ala-Ala (Table 2). These data strongly support the concept that the role of F-type ATPase would solely act to provide ATP for the P-type ATPase-dependent active transport in *H. pylori*. Surprisingly, OAS at a concentration of 0.1 mM did not cause Ala-Ala export at all, suggesting that extra factors other than P-type ATPase with ATP are involved in its transport. This redundancy in peptide uptake is thought to be of great significance in *H. pylori* physiology. Nevertheless, regarding its parasitic nature, the reduction in the nutrient uptake ability is thought to be fatal to this organism. Meanwhile, *H. pylori* among those bacteria tested exhibited as the most susceptible bacterium to these compounds. This result was already predicted, however, this unusual susceptibility seems inconsistent with its nature of broad resistance toward antibiotics [21]. Supposing that the factor offering this discrepancy in the susceptibitlity against OMP is on account of P-type ATPase inhibition, then its role in *H. pylori* seems unique.

We found that OMP did not form a disulfide bridge with free cysteine, revealing that it must be shifted to cationic OAS to become reactive. Therefore, the OMP molecule was thought to be activated during its uptake. That is, the rate of OMP activation in this process is presumably a critical factor influencing the antibiotic activity. The mechanism of OMP turnover is being studied in our laboratory. At low concentrations of the compounds, the enzyme activity was recovered upon reduction by DTT, but its activity could not be completely recovered when the membrane vesicles were treated with high concentrations of the compounds, especially OAS. From this observation, it was suggested that the mechanism of enzyme inhibition by OAS might be somehow complicated. More information is required in order to understand the effect of OAS on the membrane integrity of proteins.

The P-type ATPase family in living systems is involved in cytoplasmic cation homeostasis [12]. In bacterial cell membranes, however, this enzyme activity is not common but rather ubiquitous [13]. As mentioned before, since the P-type ATPase pool in *H. pylori* cell membranes was preferentially found as a major proton pump [40, 41], its physiological role would perhaps be different from those found in other bacterial membranes. In the literature, *H. pylori* P-type ATPase is supposed to regulate cytoplasmic pH, especially at low pH. At neutral pH, its role is thought to be the provision of electrochemical gradients on cell membranes [2, 25, 26]. Nonetheless, clear evidence has not yet been found to support this assumption. In addition, glycolysis is not well established in this bacterium [27, 32]. From our finding that the relative specific activity of F-type ATPase of *H. pylori* to that found in *E. coli* was below 0.1 [40], the overall significance of P-type ATPase activity must be counterbalancing the active transport of nutrients.

In conclusion, the data presented in this paper obviously reveals that OMP can easily be taken up by *H. pylori* cells and activated to form disulfide bridges at the catalytic domain of P-type ATPase, localized in the cytoplasm.

Following the inactivation of the enzyme, the cells become defective in the active transport for cationic minerals, nutrients, etc., leading to cell death. Taking its physiological role into consideration, P-type ATPase is suggested as an ideal target to eradicate *H. pylori in vivo*.

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