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NOTES

Effects of Cations on *Helicobacter pylori* Urease Activity, Release, and Stability

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The urease of *Helicobacter pylori* is an important antigen and appears critical for colonization and virulence. Several studies have indicated a superficial localization for the *H. pylori* urease, and the purpose of this study was to determine the effects of cations on the release and stability of urease activity from *H. pylori* cells. Incubation of partially purified *H. pylori* urease in water containing 1, 5, or 10 mM Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, EDTA, or EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] had little effect on activity. In contrast, 1 mM Fe^{3+}, Cu^{2+}, Co^{2+}, or Zn^{2+} substantially (>80%) inhibited activity, and 10 mM Fe^{2+}, Mn^{2+}, and Ni^{2+} inhibited about 30% of the activity. Addition of Ca^{2+} or Mg^{2+} markedly decreased extraction of urease from intact *H. pylori* cells by water, but 1 mM Na^{+}, K^{+}, EGTA, or EDTA each had minimal effects on release, suggesting that divalent cations have a role in attachment of urease to *H. pylori* cells. The stability of enzymatic activity at 4°C was enhanced by addition of glycerol or 2-mercaptoethanol; however, even after loss of activity, full antigenicity for human serum was retained.

Helicobacter pylori, a gram-negative bacterium that is a human gastroduodenal pathogen, produces large amounts of the enzyme urease (18). Urease is believed to facilitate colonization (7) by generating ammonia to buffer gastric acidity (19, 25); ammonia also may contribute to mucosal injury (2, 12). The enzyme is immunogenic for humans and is highly conserved among *H. pylori* strains (3, 9). The urease of *H. pylori* is a high-molecular-weight, multimeric, nickel-containing enzyme similar to other bacterial ureases (6, 8, 10, 11, 13). For these other enzymes, activity may be inhibited by a variety of cations (16, 17, 19, 20).

However, although urease represents more than 5% of the total *H. pylori* proteins and the subunits are synthesized without cleaved leader sequences, most evidence suggests that the assembled enzyme is cell surface associated (1, 6, 14, 18), in contrast to other bacterial ureases, which are cytoplasmic (19). In previous studies, we have shown that extraction of *H. pylori* cells in water releases urease (6). For other surface-associated molecules of gram-negative bacteria that are water extractable, addition of divalent cations inhibits release (5, 21, 27).

The goals of the present study were to examine the interactions of cations with *H. pylori* urease, both in terms of enzyme activity and release from cells. Moreover, since urease is an important reagent for serodiagnostic assays to detect *H. pylori* infection (3), we also sought to understand the effects of ions on enzyme stability and antigenicity.

Cells of *H. pylori* 84-183 (22), used in all experiments, were grown for 72 h on Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) at 37°C in an atmosphere containing 5% CO_2. Cells were harvested in 0.15 M NaCl (pH 7.0), centrifuged (5,000 × *g*) for 20 min, and then resuspended in either distilled water or 1 to 10 mM solutions of NaCl, KCl, $MgCl_2$, $CaCl_2$, EDTA, or EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid]. The cell suspensions were vortexed for 45 to 60 s and centrifuged (5,000 × *g*) for 30 min, and the urease activity present in the supernatant was determined. In experiments to determine the direct effects of cations on urease activity, 1 to 10 mM concentrations of $FeCl_3$, $FeCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$, $ZnCl_2$, and the salts listed above were added to the supernatants extracted with distilled water. The viability of *H. pylori* cells was assessed by preparing 10-fold dilutions of the suspensions before and after suspension in water or 0.15 M NaCl and plating in duplicate on blood agar plates. Colonies were counted after microaerobic incubation for 72 h at 37°C.

Partially purified urease preparations were prepared as previously described (6). In brief, *H. pylori* whole cells were resuspended in distilled water, vortexed for 90 s, and then centrifuged at 5,000 × *g* for 30 min, and the supernatant was used for evaluation of the stability of enzyme activity. The urease preparation was diluted in 50 mM phosphate buffer (pH 7.5) or 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) buffer (pH 7.5) and combined with 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 M NaCl, 50% glycerol, or combinations of these agents. Urease activity of preparations stored at 4°C was determined weekly for up to 25 weeks. Urease activity was assayed exactly as previously described (6). Protein concentrations of whole cells and extracts were measured using the BCA protein assay (Pierce, Rockford, Ill.).

To determine the antigenicity of *H. pylori* urease preparations after various treatments, we used an enzyme-linked immunosorbent assay (23) with the modifications described

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TABLE 1. Effects of cations and chelators on *H. pylori* urease activity[a]

Salt or chelator	% Change in urease activity at:		
	1 mM	5 mM	10 mM
KCl	−4.4 ± 6.3	−0.5 ± 3.7	−0.1 ± 5.3
NaCl	+0.1 ± 8.3	−5.2 ± 3.8	−7.6 ± 2.1
$MgCl_2$	−0.7 ± 4.3	−5.3 ± 3.7	−7.0 ± 5.4
$CaCl_2$	−5.7 ± 3.1	−7.5 ± 0.4	−13.6 ± 2.9
$MnCl_2$	−26.7 ± 8.1	−27.6 ± 6.8	−30.1 ± 0.6
$FeCl_2$	−6.6 ± 9.0	−35.2 ± 6.1	−49.2 ± 4.5
$NiCl_2$	−57.2 ± 5.1*	−88.5 ± 2.5*	−85.0 ± 4.4*
$CoCl_2$	−92.9 ± 5.3*	−88.7 ± 7.3*	−93.0 ± 4.4*
$ZnCl_2$	−90.4 ± 2.0*	−97.3 ± 0.3*	−97.7 ± 0.2*
$CuCl_2$	−99.9 ± 0.1*	−99.9 ± 0.1*	−99.6 ± 0.1*
$FeCl_3$	−99.7 ± 0.2*	−99.7 ± 0.2*	−99.7 ± 0.1*
EGTA	−2.7 ± 5.2	−5.7 ± 6.8	−5.6 ± 3.0
EDTA	+1.4 ± 4.7	−6.3 ± 3.5	−10.1 ± 4.1

[a] The specified salt or chelator in distilled water at the concentration indicated was added to an *H. pylori* urease preparation. The urease activity observed was compared with the activity observed when the same urease preparation was incubated in distilled water alone. Results shown are the means ± standard errors of the means for four replicate experiments, and those indicated by asterisks are significantly different from the values for water alone. Since multiple comparisons were being made, Bonferoni's correction was utilized, and significance was defined as $P < 0.001$.

elsewhere (24) to examine a panel of serum samples from six *H. pylori*-infected adults. For each urease preparation, 10 μg of protein per ml was applied to microtiter plate wells.

To determine whether mono-, di-, or trivalent cations or divalent cation chelators affected *H. pylori* urease activity, urease obtained by water extraction was incubated with various concentrations of these ions or chelators and activity was compared with that for incubation with water alone. The water extract is approximately fourfold enriched for urease compared with whole-cell sonicates (6). Incubation with Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, EDTA, or EGTA had minor effects, whereas Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mn^{2+}, and Zn^{2+} markedly inhibited urease activity (Table 1).

Other reports show either inhibition or stimulation of activity of other bacterial ureases by cations (15–17, 20). One explanation for inhibition by divalent cations is that they may compete with or replace the essential nickel ion (10), as has been suggested for jackbean urease (4), or may distort the active site; more than one mechanism may be present. Divalent cations that inhibit *H. pylori* urease may have value in development of specific antimicrobial therapies. We speculate that failure of calcium or magnesium to significantly inhibit may reflect dissimilarities with nickel in terms of atomic radius or charge density. The relative resistance of the urease to EDTA and EGTA is of interest since these chelators might be expected to inhibit a metalloenzyme. The lack of inhibition with millimolar EDTA concentrations suggests that either the urease polypeptide has an extremely high affinity for nickel or that this ion is protected by its location in the interior of the macromolecular complex. The slight stimulation by EDTA that was observed could be due to chelation of competing cations present at trace concentrations.

Next, we were concerned with the attachment of urease to *H. pylori* cells; extraction in water released 75.4% ± 3.9% of the urease activity from cells. After extraction, only 20.8% of the urease activity remained in the cells and specific activity present in the cellular fraction fell (Table 2). In comparison, extraction in saline also had little effect on cell viability but resulted in essentially no extraction of urease from the cells. That water extraction does not have a major effect on viability is further evidence that the release of urease is not due to cell lysis, consistent with earlier data that the extract is enriched in urease relative to that in intact cells (6) and with the observation that urease may be extracellular (1). Water extraction has been used to release other molecules, especially S-layer proteins that are external to the outer membrane of gram-negative bacteria, and addition of divalent cations can inhibit this release (5, 27).

TABLE 2. Effect of extraction on viability and urease activity of *H. pylori* whole cells

Extraction solution	Cells before extraction		Cells after extraction	
	CFU[a]	Sp act[b]	CFU	Sp act
Water	7.53 ± 1.8	24.9 ± 4.1[c]	6.84 ± 1.9	5.5 ± 1.8[c]
Saline[d]	7.75 ± 1.2	19.0 ± 0.2	7.67 ± 1.2	18.5 ± 0.1

[a] Mean log_{10} of CFU per milliliter ± standard error (mean of three experiments).
[b] Specific activity of *H. pylori* urease: micromoles of urea hydrolyzed per minute per milligram (6).
[c] $P = 0.02$ by Student's *t* test.
[d] 0.15 M NaCl.

To determine whether release of urease activity from *H. pylori* cells in water could be modified, extractions were done in the presence of various ions or chelators (Fig. 1). At concentrations ≥5 mM, EGTA and all cations studied significantly inhibited release of urease activity from *H. pylori* cells. At 1 mM concentrations, only the divalent cations had a substantial effect. In contrast, EDTA, in either concentration tested, did not significantly inhibit urease release (Fig. 1). Ca^{2+} and Mg^{2+} inhibited release of urease activity in a dose-dependent fashion to nearly identical degrees, with concentrations lower than 0.1 mM showing minimal effect, whereas 1 mM produced 79 and 77% inhibition of urease release, respectively.

The dose-dependent release inhibition by Ca^{2+} and Mg^{2+} supports the hypothesis that these ions play a role in attachment of urease to the *H. pylori* cell surface, analogous to their roles in maintaining the integrity of S layers of other gram-negative bacteria (5, 27). Alternatively, the fact that these ions stabilize the outer membranes of gram-negative organisms may be important (26). Such stabilization of the *H. pylori* outer membrane may prevent removal of mem-

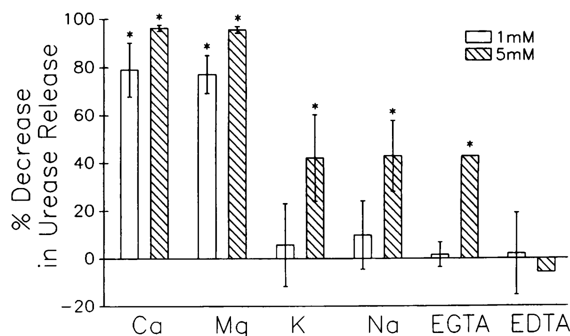

FIG. 1. Effects of cations and chelators (at 1 and 5 mM) on release of urease activity from *H. pylori* cells in comparison with extraction in water alone. Results shown are means ± standard errors of three replicate experiments. Asterisks indicate significant differences at $P < 0.05$ in comparison with water alone.

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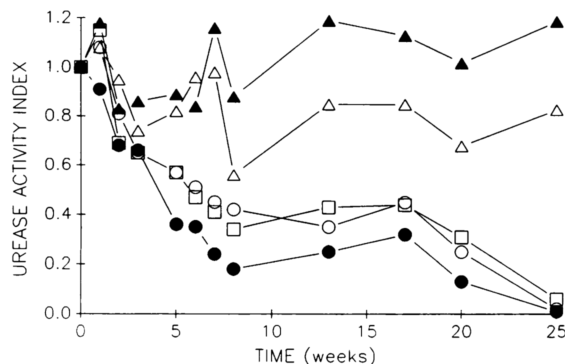

FIG. 2. Stability of *H. pylori* urease activity in water extracts at 4°C in the presence of different stabilizers. Results shown are ratios of the observed activity at specified time points in relation to the initial activity in the sample and are the means of two replicate determinations. Symbols: ○, no stabilizer (water extract alone); △, 50% glycerol; ▲, 2-mercaptoethanol (1 mM); □, EDTA (1 mM); ●, HEPES buffer (50 mM), pH 7.5.

brane-associated urease or leakage of urease from periplasmic contents. The effects of other ions that occurred at high molarities (≥5 mM) probably are nonspecific.

Because *H. pylori* urease may have utility as a reagent in the biotechnology industry, we next assessed its activity in the presence of several chemicals that have been described as enzyme stabilizers (19). After extraction in water and storage at 4°C, the activity of *H. pylori* urease progressively decreased over a 20-week period (Fig. 2). Addition of 50 mM HEPES buffer or 1 mM EDTA did not reduce the decline in activity; however, both 2-mercaptoethanol and glycerol significantly preserved urease activity during prolonged storage. Other experiments that analyzed other time points showed similar results (data not shown). The decline in urease activity was not pH dependent, since it was not affected by buffering, nor was it due to cation contamination since EDTA was not useful. Glycerol stabilizes other enzymes (19), perhaps because the high osmolality of glycerol-containing solutions promotes the retention of hydrophobic interactions necessary for enzyme activity. The general mechanism by which 2-mercaptoethanol stabilizes enzyme activity is by preventing oxidation (19), but the mechanism for its effects on *H. pylori* urease is not known. In any event, virtually all enzymatic activity can be retained for prolonged periods when appropriate conditions are utilized.

We then investigated whether antigenicity of *H. pylori* urease assessed by recognition by serum antibodies from *H. pylori*-infected persons was related to enzymatic activity in preparations maintained in the stabilizers mentioned above. Despite significant variation in urease activity at 6 weeks in the different stabilizers (89.7 versus 34.1% decrease, P = 0.006), antigenicity was highly conserved (98 versus 98.5%). Thus, even small changes in conformation may affect enzyme activity but may have little impact on affinity of the high-titer polyclonal antibodies present in naturally infected persons (6). Use of monoclonal antibodies may permit better definition of epitopes lost as enzymatic activity diminishes.

In conclusion, particular cations affect the activity of *H. pylori* urease as well as its attachment to the cell surface. Further characterization of these interactions may lead to new avenues for therapy as well as improved diagnostics based on the integrity of the enzyme.

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