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Transport Kinetics and Selectivity of *Hp*UreI, the Urea Channel from *Helicobacter pylori*

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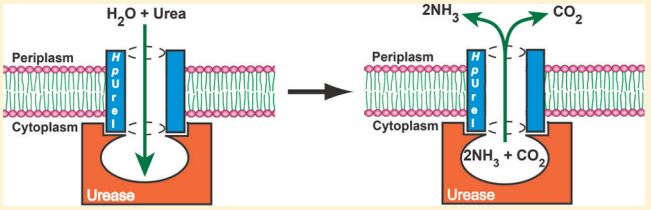
ABSTRACT: *Helicobacter pylori*'s unique ability to colonize and survive in the acidic environment of the stomach is critically dependent on uptake of urea through the urea channel, *Hp*UreI. Hence, *Hp*UreI may represent a promising target for the development of specific drugs against this human pathogen. To obtain insight into the structure−function relationship of this channel, we developed conditions for the high-yield expression and purification of stable recombinant *Hp*UreI. Detergent-solubilized *Hp*UreI forms a homotrimer, as determined by chemical cross-linking. Urea dissociation kinetics of purified *Hp*UreI were determined by means of the scintillation proximity assay, whereas urea efflux was measured in *Hp*UreI-containing proteoliposomes using stopped-flow spectrometry to determine the kinetics and selectivity of the urea channel. The kinetic analyses revealed that urea conduction in *Hp*UreI is pH-sensitive and saturable with a half-saturation concentration (or $K_{0.5}$) of ∼163 mM. The extent of binding of urea by *Hp*UreI was increased at lower pH; however, the apparent affinity of urea binding (∼150 mM) was not significantly pH-dependent. The solute selectivity analysis indicated that *Hp*UreI is highly selective for urea and hydroxyurea. Removing either amino group of urea molecules diminishes their permeability through *Hp*UreI. Similar to urea conduction, diffusion of water through *Hp*UreI is pH-dependent with low water permeability at neutral pH.



Urea is an important biological molecule with diverse functions. While urea is a nitrogen source for bacteria, mammals excrete it as a less toxic byproduct of nitrogen metabolism.[1] In fish gills and mammalian kidneys, urea also functions as an osmolyte.[2] In *Helicobacter pylori*, a pathogenic bacterium responsible for several gastroduodenal disorders, including chronic active gastritis, peptic ulcer disease, and gastric cancer, urea plays a unique and essential role in its colonization of the acidic stomach.[3−6] Although *H. pylori* is a neutralophilic bacterium that grows optimally at pH 7.0, the presence of urea allows it to grow below pH 4.0.[7]

H. pylori uses a unique mechanism to take advantage of urea to neutralize the acidic stomach.[8,9] In this mechanism, urea is taken up from the stomach environment via an inner membrane urea channel (*Hp*UreI). A cytoplasmic urease then hydrolyzes imported urea into NH_3 and CO_2, which subsequently diffuse into the periplasmic space. There, NH_3 neutralizes the acid, and CO_2 is converted into HCO_3^- (by a membrane-associated carbonic anhydrase) that provides a buffering range of pH 6.1 (Figure 1).[9] This mechanism allows *H. pylori* to maintain a proton-motive force necessary for cell growth in the gastric environment.[2] The urea concentration in gastric juice, like that in blood, is 3−5 mM, which is enough to support bacterial survival.[2,10,11] *Hp*UreI is essential for *H. pylori* to neutralize the acid and survive in the stomach.[8,10,12,13]

*Hp*UreI expression in *Xenopus* oocytes has demonstrated that the protein is a passive, pH-gated, and temperature-independent urea channel.[8,14] The acid sensitivity of the urea channel is essential for cytoplasmic pH homeostasis in *H. pylori*.

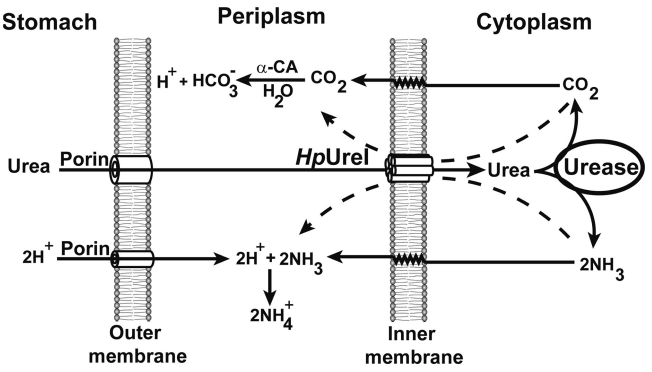


Figure 1. Urea channel, *Hp*UreI, plays a central role in the mechanism of acid acclimation by *H. pylori*. The conduction of urea from the periplasm into the cytoplasm is facilitated by the *Hp*UreI. The cytoplasmic urease consequently hydrolyzes urea to CO_2 and NH_3, which eventually reach the periplasm by simple diffusion through the lipid bilayer or by conduction through the *Hp*UreI channel, as recently suggested.[36] In the periplasm, NH_3 neutralizes the acidic pH and CO_2 (which is converted into HCO_3^- by membrane-associated α-carbonic anhydrase, α-CA) buffers the periplasm at pH ∼6.1.

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When the pH is acidic (<5.0), the bacterium requires urea to produce NH_3 and CO_2, and the channel remains open to facilitate urea uptake. On the other hand, at neutral pH, urease activity is not required, and the channel is closed to prevent toxicity caused by cytoplasmic alkalization. With a molecular mass of 21.7 kDa (195 amino acids), *Hp*UreI is one of the smallest pH-gated channels known.

There are multiple urea transport systems facilitating the permeation of urea across cell membranes, including, but not limited to, urea transporters (UT)[15,16] and acid-sensitive urea channels (UreI).[8] UT and *Hp*UreI are very different in terms of sequence and topology. Unlike *Hp*UreI, which has six transmembrane domains, UT consists of 12 transmembrane domains.[17] Recent functional and structural studies of UT family members have indicated that they employ a channel-like mechanism.[17−20] Compared to UT, our knowledge of the structure and mechanism of *Hp*UreI channels is very limited.

In this research, we heterologously expressed and purified *Hp*UreI. The purified *Hp*UreI was reconstituted into liposomes, and a stopped-flow assay was used to determine the kinetics of solute conduction by *Hp*UreI. Our results indicate that *Hp*UreI is a trimeric channel with high selectivity for urea and hydroxyurea. *Hp*UreI also conducts water in a pH-dependent manner, indicating that water and urea share a common conduction pathway. Although the conduction of urea is pH-sensitive, the binding of urea by *Hp*UreI is pH-independent. Finally, we propose a model that takes into account these and previous results.

MATERIALS AND METHODS

Expression and Purification of *Hp*UreI. The genomic DNA of *H. pylori* strain 26695 [purchased from American Type Culture Collection (ATCC)] was used as the template for the polymerase chain reaction amplification of the *ureI* gene. The amplified fragment was digested with suitable restriction enzymes and ligated into a pET29b vector, which had been digested with the same restriction enzymes. To remove the His tag during protein purification, a TEV cleavage site (ENLYFQG) was engineered between the *ureI* gene and the His tag.

The expression vector was transformed into *Escherichia coli* BL21 Rosetta 2 (DE3) competent cells. Protein expression under various growth conditions was evaluated by detection of the His tag using HisProbe-HRP (Pierce/ThermoScientific). We achieved optimal expression by inducing cell cultures with 0.1 mM IPTG at an OD_{600} of 0.6 and growing the cells overnight at 25 °C.

Cells were harvested by centrifugation and resuspended in lysis buffer [25 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, and 4 mM βME (β-mercaptoethanol) supplemented with fresh PMSF and DNase I]. Cells were lysed by two passes through an EmulsiFlex-C3 homogenizer (Avestin), and the membrane fraction was harvested by centrifugation at 138000*g* for 1 h. The protein was extracted from the membrane by overnight agitation in extraction buffer [25 mM Tris (pH 7.5), 500 mM NaCl, 40 mM decyl β-D-maltoside (DM, Anatrace), 20 mM imidazole, and 4 mM β-ME] at 4 °C. The solubilized protein was loaded onto a Ni^{2+} affinity column (Ni-NTA resin, Qiagen), which was pre-equilibrated with nickel column buffer [25 mM Tris (pH 7.5), 500 mM NaCl, 4 mM DM, 20 mM imidazole, and 4 mM β-ME]. The resin was washed first with 20 mM imidazole and then with 50 mM imidazole in nickel column buffer. The protein was eluted with 400 mM imidazole in nickel column buffer. Imidazole was removed using Econo-Pac 10DG desalting columns (Bio-Rad), and the His tag was removed by overnight incubation with TEV protease at 4 °C. TEV protease and undigested proteins were removed by passing over a Ni^{2+} affinity column and collecting the flow through. The protein was injected onto a Superose 6 10/300 GL column (GE Healthcare) with a mobile phase of 20 mM HEPES (pH 7.5), 200 mM NaCl, 4 mM DM, and 2 mM dithiothreitol (DTT). The symmetric peak of *Hp*UreI was collected, and its purity was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The size homogeneity of the purified protein was routinely confirmed by reinjection into the size-exclusion chromatography column. All chemicals and buffers were purchased from Sigma.

Cross-Linking Experiments. Glutaraldehyde was used to cross-link *Hp*UreI monomers. For the cross-linking reaction, *Hp*UreI was purified in phosphate buffer [20 mM KP_i (pH 7.4), 200 mM NaCl, 10% glycerol, and 4 mM DM]. To initiate the cross-linking reaction, glutaraldehyde was added to a final concentration of 100 mM. To monitor the progress of the reaction, aliquots (25 μL) were removed from the reaction mixture at various time points of 1, 2.5, 5, 20, 60, and 120 min, and the reaction was quenched by the addition of 1 M Tris (pH 7.5) (final concentration of 142 mM). The samples were analyzed on a 4 to 20% gradient SDS−PAGE gel visualized by silver staining.

Reconstitution of Proteoliposomes. *E. coli* polar lipids (purchased as a chloroform solution from Avanti Polar Lipids, Inc.) were dried under N_2, washed twice with pentane, and further dried with a speedvac for 1 h. The dried lipid was resuspended in 20 mM HEPES (pH 7.5), 200 mM NaCl, and 2 mM DTT to a final concentration of 10 mg/mL. The lipid suspension was sonicated to clarity and diluted with DM buffer [~7 mM DM, 20 mM HEPES (pH 7.5), 200 mM NaCl, and 2 mM DTT] to 2.5 mg/mL lipid and incubated for 30 min at room temperature. Protein was added to a final lipid:protein weight ratio of 100 (2 mg of lipid per 0.02 mg of protein) and incubated for 1 h at room temperature. Detergent was removed by incubation with Biobeads SM-2 (Bio-Rad). The vesicles were extruded (through a 0.4 μm filter) multiple times to achieve homogeneity. The vesicles were harvested by centrifugation at 80000*g* for 1 h at 4 °C. Monodispersity and size distributions of the vesicles were routinely verified by dynamic light scattering, and the vesicle diameter was generally 100 ± 10 nm. Identical procedures were followed to prepare control liposomes in the absence of protein. All samples were assayed within a day of preparation.

To measure solute permeability coefficients at pH 5.0, the buffer in all solutions used to prepare proteoliposomes was replaced with 20 mM sodium acetate (pH 5.0). To determine the pH profile of *Hp*UreI, the vesicles were prepared in the pH range of 4.0−7.5 using a 20 mM citrate-phosphate buffer adjusted to the various pH values.

Solute Permeability Measurements. Permeabilities for urea or urea analogues were measured using a stopped-flow spectrometer (Applied PhotoPhysics SX20) with a dead time of ≤1 ms. To determine the solute selectivity of *Hp*UreI, the permeability assays were conducted at pH 5.0, where the channel is conductive. Each solute was loaded into the vesicles (control liposomes or *Hp*UreI proteoliposomes) by overnight incubation in assay buffer [20 mM sodium acetate (pH 5.0) and 100 mM NaCl] supplemented with 200 mM urea or urea analogues. The vesicle suspension was abruptly mixed (1:1)

with an assay solution supplemented with sucrose (the nonpermeant osmolyte) to create an isoosmotic solution. The osmolality of each buffer was determined using a Vapro vapor pressure osmometer (Wescor, Inc.). When the compounds were mixed, the external concentration of the permeant solute was reduced by half, generating a solute concentration gradient that drove solute efflux. Solute efflux was followed by water efflux, leading to the reduction of the vesicle volume, which caused an increase in the intensity of scattered light measured at 440 nm (λ).[21,22] All the permeability assays were conducted at 10 °C. The rate constant of solute diffusion was determined by exponential curve fitting of the average of five to eight traces. The measured rate constants were used to determine permeability coefficients based on Mathai and Zeidel equations.[23] Each data point is the average of three to five independent permeability measurements.

pH−Activity Profile of *Hp*UreI. The pH profile of *Hp*UreI activity was determined from pH 4 to 7.5. For each pH value, the proteoliposomes were independently prepared using 20 mM citrate-phosphate buffer adjusted to the desired pH. The standard deviation of each data point is a result of three to five experiments. The urea permeability of control liposomes was also measured at each pH, to determine the background permeabilities.

Measurement of the Apparent $K_{0.5}$ of *Hp*UreI. To measure the kinetic parameters for urea conduction by *Hp*UreI, the urea flux was measured at various concentration gradients of urea. To that end, *Hp*UreI proteoliposomes were loaded with various concentrations of urea by overnight incubation in assay buffer (20 mM citrate-phosphate pH 5.0 plus 100 mM NaCl) containing 50 mM to 800 mM urea. The vesicle suspension was rapidly mixed (1:1) with an assay solution containing iso-osmotic concentrations of sucrose. The efflux of urea was measured as an increase in the intensity of scattered light at 440 nm.

Water Permeability Measurements. The water permeability of *Hp*UreI was measured by rapidly mixing the proteoliposome (or liposome for control experiments) suspension in assay buffer with a hyperosmotic solution of 500 mM sucrose at 10 °C. The sucrose osmotic gradient drove water efflux, and vesicle shrinkage was measured as an increase in the intensity of scattered light at 440 nm. Typically, five to eight traces were averaged and used for exponential curve fitting to measure the rate constant of osmotic water efflux. All the data points represent the average of three to five permeability measurements from independent proteoliposome preparations. Osmotic water permeabilities were calculated from the measured rate constants using approaches of Mathai and Zeidel.[23]

***Hp*UreI Scintillation Proximity Binding Assay.** Binding of 181 μM [^{14}C]urea (55 mCi/mmol, American Radiolabeled Chemicals, Inc.) to recombinant *Hp*UreI was assayed by means of the scintillation proximity assay (SPA).[17,24,25] Cu^{2+}-coated YSi SPA beads (Perkin-Elmer catalog no. RPNQ00096) were diluted to a concentration of 2.5 mg/mL in a 150 mM Tris, Mes (pH 7.5 or 5.0), 50 mM NaCl, 20% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma Co.), 0.1% *n*-dodecyl β-D-maltopyranoside mixture. His-tagged recombinant protein (at 250 ng per assay) and [^{14}C]urea were added simultaneously to the YSi SPA bead mix in the presence or absence of increasing concentrations of nonlabeled urea in individual wells of clear-bottom, white-wall 96-well plates (Greiner catalog no. 655095). Plates were incubated in the dark at 4 °C with vigorous shaking on a vibrating platform for 15 min before their contents were counted in the SPA mode of a Wallac 1450 MicroBeta plate PMT counter. Nonspecific background binding activity was assayed in the presence of 800 mM imidazole (as imidazole competes with the His-tagged proteins) in control samples for all conditions tested and subtracted from the total binding activity (detected as counts per minute) to obtain the UreI-specific binding activity. Data points represent the mean ± standard error of triplicate determinations. Data fits of kinetic analyses were performed using nonlinear regression algorithms in Prism, and errors represent the the standard error of the mean of the fit.

RESULTS AND DISCUSSION

Cloning, Expression, and Purification of *Hp*UreI. Several constructs of the *ureI* gene in different pET vectors, including pET29b, pET28b, and pET27b, were built and screened to identify the best conditions for overexpression of *Hp*UreI. In addition, several growth and expression variables were screened to determine the best conditions of *Hp*UreI overexpression. The yield was estimated to be 0.3−0.5 mg of protein/L of medium.

Screening of detergent solubilization showed that dodecyl maltoside (DDM), undecyl maltoside (UDM), decyl maltoside (DM), and lauryldimethylamine oxide (LDAO) all efficiently extract *Hp*UreI, and the purification of the *Hp*UreI in each of these detergents yielded a stable protein. We used DM for purification and all the functional studies.

The DM-extracted *Hp*UreI was purified using Ni^{2+} affinity purification, followed by TEV protease digestion to remove the His tag. Undigested protein, along with TEV protease (which also carries a C-terminal His tag), was removed by a second Ni^{2+} affinity column. The TEV-digested *Hp*UreI was further purified by size-exclusion chromatography (Figure 2A). *Hp*UreI eluted from the Superose 6 10/300 GL column as a single peak with a retention volume of 16.3 mL corresponding to a molecular mass of <150 kDa. SDS−PAGE analysis confirmed the purity of the protein (Figure 2B, lane 1).

Oligomeric State of Detergent-Solubilized *Hp*UreI. To determine the oligomeric state of detergent-solubilized *Hp*UreI, we performed cross-linking analysis. To this end, the protein was treated with glutaraldehyde for various time periods, and the products of the reaction were analyzed by 4 to 20% gradient SDS−PAGE (Figure 2B, lanes 2−7). As the reaction progressed, the ∼20 kDa band (representing the monomeric form of *Hp*UreI) disappeared and two bands migrating at ∼40 and ∼60 kDa emerged, representing the dimeric and trimeric forms of the protein, respectively. Bands larger than trimer were not observed even after exposure to the cross-linker for 24 h. The glutaraldehyde cross-linking analysis indicates that detergent-solubilized *Hp*UreI exists as homotrimer.

Most of the membrane channels for neutral solutes form multimeric assemblies in the biological membranes, even though each monomer can be an independent functional unit. Examples include water channels[22,26] and ammonia channels.[27] The urea transporter from the bacterium *Desulfovibrio vulgaris* also exists as homotrimer in the crystal structure.[17] The trimeric assembly could represent the active form of *Hp*UreI in the cell membrane.

The results of both co-immunoprecipitation analysis[28] and immunoelectron microscopy experiments[29] showed that cytoplasmic urease interacts with *Hp*UreI. The functional unit

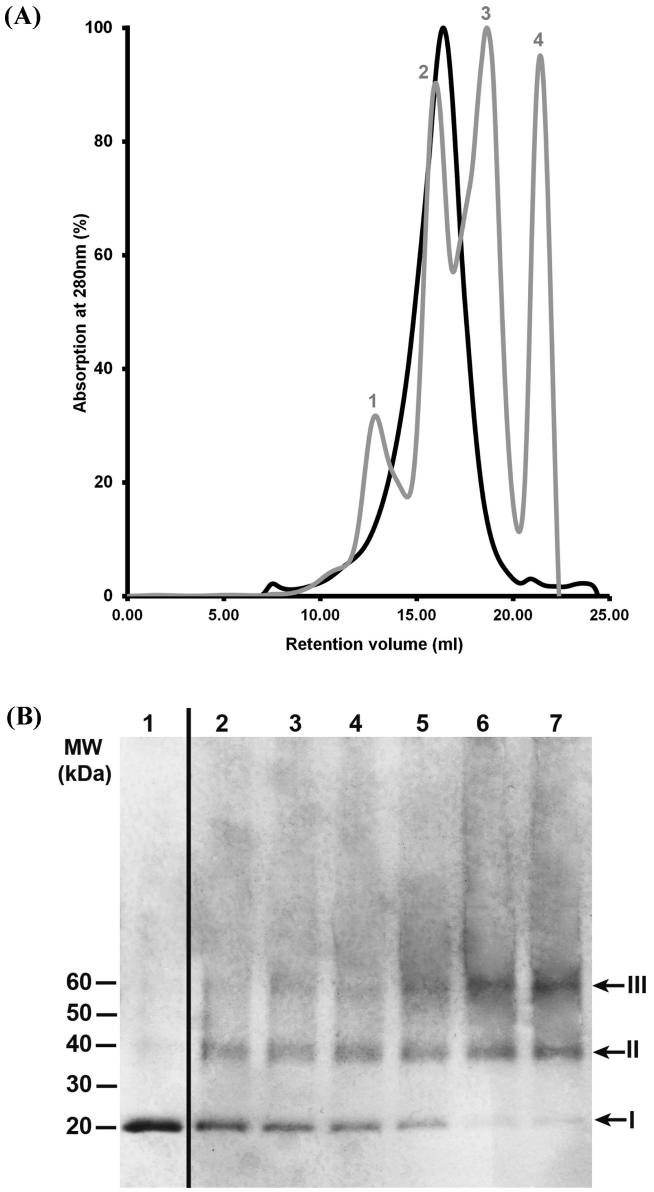

Figure 2. *Hp*UreI purification in DM. (A) Size-exclusion chromatogram of purified *Hp*UreI. The black line represents the elution of *Hp*UreI, and the gray line is that of protein standards, including (1) thyroglobulin (670 kDa), (2) γ-globulin (158 kDa), (3) myoglobin (17 kDa), and (4) vitamin B_{12} (1.35 kDa). (B) Chemical cross-linking analysis of *Hp*UreI. The purified *Hp*UreI (lane 1) was incubated with glutaraldehyde for 1 (lane 2), 2.5 (lane 3), 5 (lane 4), 20 (lane 5), 60 (lane 6), or 120 min (lane 7) before the addition of Tris to quench the reaction. The results were analyzed using a 4 to 20% gradient SDS–PAGE gel visualized by silver staining. The band at 60 kDa, which corresponds to the trimeric assembly of *Hp*UreI, was formed by longer exposure to glutaraldehyde.

of *H. pylori* urease includes two different subunits (α and β).[30] The active urease from *H. pylori* consists of three (αβ) heterodimers forming a trimeric assembly, $(\alpha\beta)_3$, although the crystal packing of *H. pylori* urease shows an $[(\alpha\beta)_3]_4$ dodecameric assembly.[31] On the basis of our results, one can conclude that *Hp*UreI associates with urease through trimer–trimer interactions. This can be similar to the interaction of trimeric AmtB (an ammonia channel) with trimeric GlnK (a soluble protein that regulates AmtB activities).[32,33]

Urea Permeability Determination Using a Stopped-Flow Assay. A stopped-flow-based proteoliposome assay was used to measure urea permeability. *Hp*UreI-containing proteoliposomes or control liposomes were loaded with urea by incubation in solute-containing buffer. In a stopped-flow spectrometer, the vesicle suspension was abruptly mixed with an equal volume of an iso-osmotic solution of buffered sucrose. This results in the efflux of urea along its concentration gradient, which consequently initiates water efflux causing shrinkage of the vesicles. We determined the rate of vesicle shrinkage, measured by the change in intensity of the scattered light at 440 nm, as a function of the rate of urea efflux (Figure 3). Loading vesicles with self-quenching concentrations of carboxyfluorescein is another common method for monitoring the volume changes.[27,34] However, carboxyfluorescein (CF) is a pH-sensitive dye whose fluorescence is much reduced below pH 7.0, restricting its usage under acidic conditions. Furthermore, these two methods yielded identical urea permeability measurements at neutral pH (data not shown). Therefore, we chose to use light scattering measurements.

Our assays show that the urea permeability coefficients of liposomes without protein at pH 7.5 and 5.0 are similar [$(0.032 \pm 0.007) \times 10^{-6}$ and $(0.033 \pm 0.008) \times 10^{-6}$ cm/s, respectively (Figure 3, green and red traces)]. The urea permeability coefficient of *Hp*UreI proteoliposomes at pH 7.5 is $(0.058 \pm 0.013) \times 10^{-6}$ cm/s, which is very close to that of control liposomes at either pH (Figure 3, blue trace). However, *Hp*UreI proteoliposomes at pH 5.0 have a urea permeability coefficient of $(21.0 \pm 0.83) \times 10^{-6}$ cm/s (Figure 3, black trace), indicating a significant increase (>600-fold) with respect to the control liposome permeability.

pH–Activity Profile of *Hp*UreI. Using the oocyte expression system, *Hp*UreI was shown to be a pH-sensitive channel with its highest activity at acidic pH.[8,35] Based on our results, *Hp*UreI displays a significant increase in the level of urea conduction

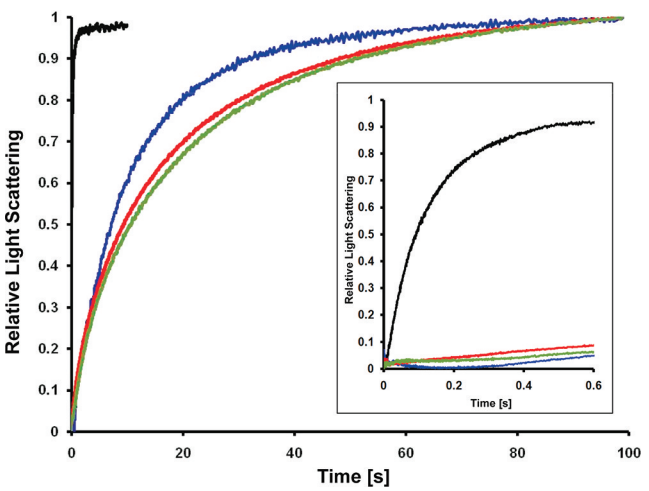

Figure 3. Time course of urea flux in *Hp*UreI proteoliposomes. The vesicles were loaded with urea and abruptly diluted in an iso-osmotic buffer using a stopped-flow spectrometer. This initiated urea efflux down its concentration gradient, which was followed by water efflux. The water efflux results in the shrinkage of vesicles that was monitored by light scattering. The traces for control liposomes (without protein) at pH 7.5 and 5.0 are colored green and red, respectively. The traces for *Hp*UreI proteoliposomes at pH 7.5 and 5.0 are colored blue and black, respectively. The inset shows a close-up of the traces during the initial 0.6 s.

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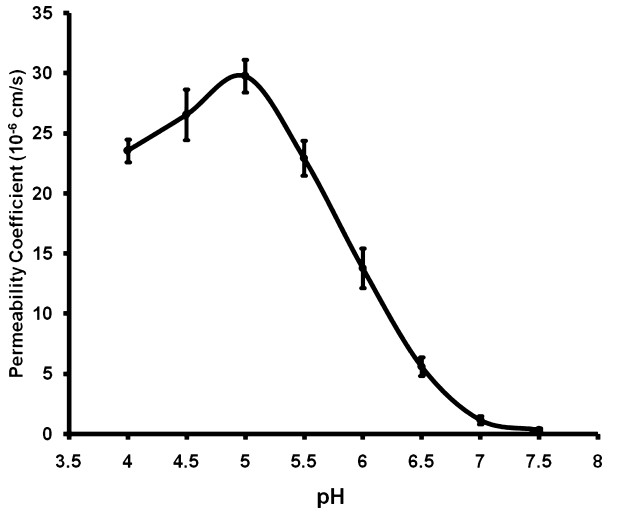

Figure 4. Profile of the pH dependence of urea permeability in *Hp*UreI proteoliposomes (mean ± standard error; n = 3−5). *Hp*UreI proteoliposomes were analyzed at various pH values ranging from 4.0 to 7.5. The pH of half-maximal activation was determined to be 5.9.

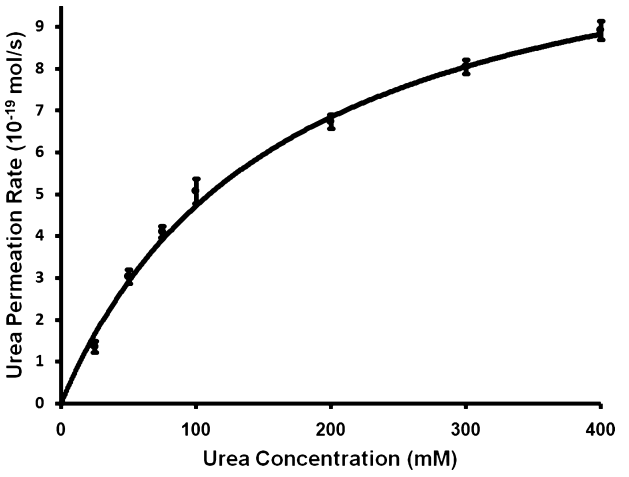

Figure 5. Kinetics of urea conduction by *Hp*UreI in reconstituted proteoliposomes. Urea permeation rate in *Hp*UreI proteoliposomes were measured at various urea concentrations. Each data point represents the mean ± standard deviation of three to five separate measurements. All the measurements were taken at pH 5.0, which is the active pH for the channel. To calculate the apparent $K_{0.5}$ (half-saturation constant), the curve was fitted with the Michaelis−Menten equation.

(more than 350-fold) when the pH decreases from 7.5 to 5.0 [$(0.058 \pm 0.013) \times 10^{-6}$ cm/s vs $(21.0 \pm 0.83) \times 10^{-6}$ cm/s (Figure 4)]. Previous results have shown that the rate of uptake of urea into *Hp*UreI-expressing *Xenopus* oocytes at pH 5.0 was only 6−10-fold higher than that in either injected oocytes at pH 7.5 or noninjected oocytes at either pH.[8] The lower activities observed in *Xenopus* oocyte expression may be due to the instability of prokaryotic *Hp*UreI in this eukaryotic expression system. However, previous in vivo assays (using oocytes)[35] and our in vitro pH profile assay show that a decreasing pH activates urea conduction by *Hp*UreI and that the pH of the half-maximal activation is ~5.9 ($pH_{0.5}$ = 5.9) (Figure 4). The periplasmic domains are thought to be involved in the pH gating of *Hp*UreI.[14] The $pH_{0.5}$ of 5.9 is similar to the pK_a of the histidine residue (6.0), suggesting these residues in the periplasmic domains may be involved in the pH gating mechanism of *Hp*UreI.

Kinetics Measurements of Urea Conduction by *Hp*UreI. The stopped-flow proteoliposome assay was used to determine the kinetics of urea conduction by *Hp*UreI. Proteoliposomes containing *Hp*UreI were loaded with various urea concentrations, and the efflux of urea was measured at pH 5.0 under iso-osmotic conditions (Figure 5). The data were fitted to the Michaelis−Menten equation, and the half-saturation concentration for urea permeation by *Hp*UreI (apparent $K_{0.5}$) was determined to be 163 ± 20 mM. Histidine apparent $K_{0.5}$ for urea conduction in *Hp*UreI is very close to previously reported values for UTs (218 mM for mammalian erythrocyte UT-B[18] and 182 mM[19] or 104 ± 9.96 mM[20] for bacterial *Ap*UT). It seems, despite their differences in sequence and topology, UT and UreI families may share similar mechanisms of solute conduction. Consistent with our results, Weeks et al. showed that a concentration gradient up to 100 mM does not saturate the solute conduction.[8] We also observed that the saturation of the solute conduction occurs only when the urea concentration gradient is above 400−500 mM, which is considerably greater than the physiological concentration of urea in the stomach (3−5 mM).

Solute Selectivity of *Hp*UreI. Solute selectivity is one of the most distinguishing features of a channel protein. Solute selectivity of *Hp*UreI has not been thoroughly investigated. Stopped-flow light scattering measurements were used to investigate the conduction of various urea analogues through *Hp*UreI. The permeability coefficient for each solute was determined for a quantitative comparative analysis. All the permeability assays were performed at pH 5.0 where the channel is conductive. Urea analogues were loaded into liposomes in a manner similar to that of urea.

Our results show that urea and hydroxyurea have the highest permeability coefficients among the solutes examined (Figure 6). This indicates that attachment of a hydroxyl group on the amino groups does not have any effect on solute conduction in *Hp*UreI.

Thiourea (the =O of urea was replaced by =S) diffuses through *Hp*UreI with a ~62% decrease in its permeability coefficient [$(21 \pm 1.7) \times 10^{-6}$ cm/s for urea vs $(7.95 \pm 1.97) \times 10^{-6}$ cm/s for thiourea (Figure 6)]. Contradictory with respect to a previous report suggesting that *Hp*UreI does not conduct thiourea,[8] our results indicate that *Hp*UreI does increase thiourea permeability with respect to the control liposome.

The significance of the two amino groups of the urea molecule was determined by replacing one of the amino groups with H (formamide), CH_3 (acetamide), or CH_2CH_3 (propionamide). These solutes diffuse through the lipid bilayer

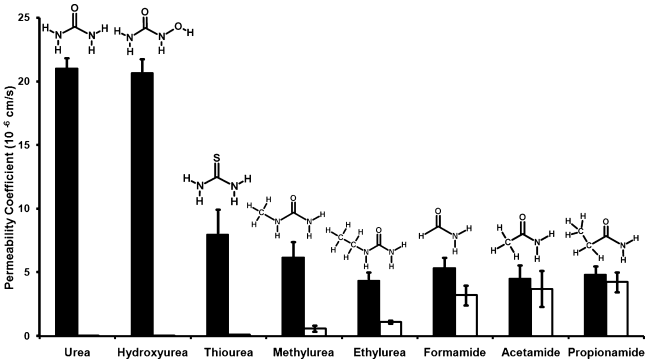

Figure 6. Solute permeability coefficients of *Hp*UreI proteoliposomes (black bars) and control vesicles (white bars) (mean ± standard error; n = 3−5). All the experiments were performed at pH 5.0.

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and consequently display permeability through control liposomes. Our results show that the permeability of these three solutes through *Hp*UreI proteoliposomes is not significantly higher than that of control liposomes (Figure 6). Therefore, removing one of the amino groups almost abolishes the ligand's permeability. This indicates the presence of both amino groups is necessary for ligand recognition and conduction by *Hp*UreI.

To determine the steric exclusion properties of the urea channel, the permeability of ligands with methyl or ethyl substituents on the amino groups was evaluated. The proteoliposome assays show that the permeabilities of methyl- and ethylurea are 4-fold lower than that of urea (Figure 6). Overall, our results indicate that the urea channel is highly selective for urea and hydroxyurea. Moreover, the solute selectivity of *Hp*UreI resembles that of UT. These results imply similarity in the solute pathways of UTs and *Hp*UreI channels.

Water permeability through *Hp*UreI proteoliposomes was measured by monitoring changes in vesicles volume due to external osmotic pressure at two pH values. For this assay, the empty vesicles were abruptly mixed with a solution of 500 mM sucrose and changes in volume were monitored on the basis of light scattering. At pH 5.0, the osmotic water permeability coefficient of *Hp*UreI proteoliposomes displayed a 6.6-fold increase [$(52.1 \pm 2.1) \times 10^{-6}$ cm/s] compared to that of control vesicles [$(8.0 \pm 1.9) \times 10^{-6}$ cm/s], indicating that water diffuses through the channel (Figure 7). The osmotic water permeability coefficient of *Hp*UreI at pH 7.5 was ~2.5-fold higher [$(18.4 \pm 1.3) \times 10^{-6}$ cm/s] than that of control liposomes [$(7.5 \pm 2.2) \times 10^{-6}$ cm/s]. At pH 7.5, where the channel is impermeable to urea, water permeability decreases significantly [$(52.1 \pm 2.1) \times 10^{-6}$ cm/s at pH 5.0 vs $(18.4 \pm 1.3) \times 10^{-6}$ cm/s at pH 7.5]. These results show that water permeability in *Hp*UreI is pH-sensitive and suggest that water and urea share a common conduction pathway.

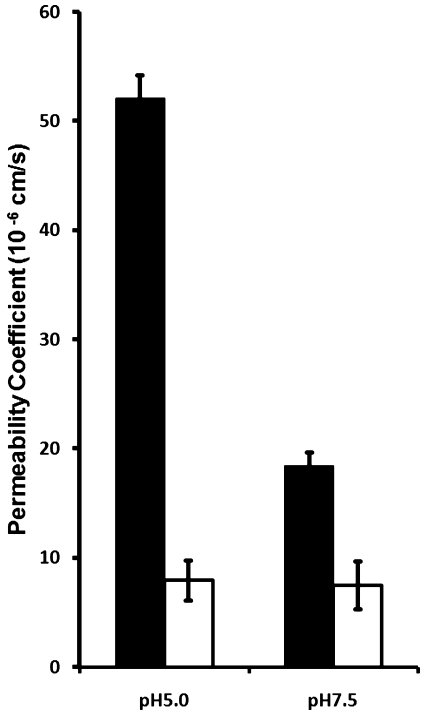

Figure 7. Osmotic water permeability of *Hp*UreI proteoliposomes (black bars) and control vesicles (white bars) at pH 7.5 and 5.0 (mean ± standard error; n = 3–5).

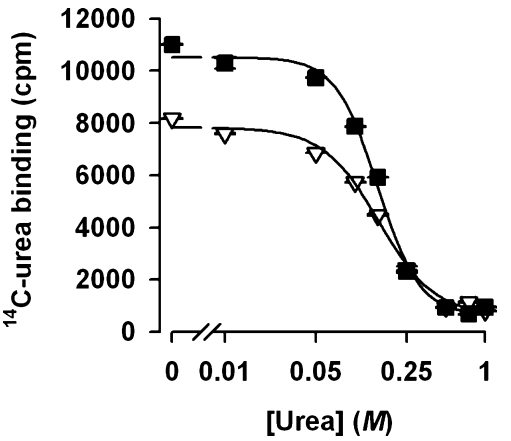

Figure 8. Equilibrium binding of [^{14}C]urea to *Hp*UreI determined with the scintillation proximity assay (SPA). Binding of 181 μM [^{14}C]urea (55 mCi/mmol) to 250 ng of recombinant His-tagged *Hp*UreI was measured at pH 5.0 (■) or 7.5 (▽) in the presence of increasing concentrations of nonlabeled urea. The progressive loss of the *HP*UreI-specific SPA-based signal (expressed as counts per minute), indicative of the displacement of bound [^{14}C]urea, was plotted as a function of nonlabeled urea concentration. Fitting the data to the Hill equation revealed a half-maximal inhibition constant (IC_{50}) of 149.4 ± 22.7 mM with a Hill coefficient of 2.7 ± 0.37 at pH 5.0 and

Binding Affinity of Urea for *Hp*UreI. The kinetics of binding of *Hp*UreI with urea was measured at pH 5.0 and 7.5 to determine how the changes in pH affect the binding of urea to the channel. The copper chelate affinity-based scintillation proximity assay (SPA) was used to directly measure the binding of [^{14}C]urea to the detergent-solubilized *Hp*UreI channel. The SPA has been used previously to measure the affinity of various ligands for their cognate transporters and channels, even when the affinity is relatively low.[17,24] Competition between binding of 181 μM [^{14}C]urea and nonlabeled urea revealed half-maximal inhibition constants (IC_{50}) of 153 ± 34 and 149 ± 23 mM at pH 5.0 and 7.5, respectively (Figure 8). This apparent affinity for urea binding is consistent with the apparent $K_{0.5}$ value for urea conduction determined using the proteoliposome assay ($K_{0.5}$ = 163 ± 20 mM). Notably, however, whereas the apparent urea affinity is independent of pH, binding of 181 μM [^{14}C]urea at pH 5.0 was ~35% higher than that observed at pH 7.5. In addition, fitting the data to the Hill equation revealed a Hill coefficient (a measure of the cooperativity of ligand binding by enzymes) of 2.7 ± 0.37 at pH 5.0, whereas the Hill coefficient at pH 7.5 was found to be 2 ± 0.32.

There are significant differences in the K_d values between the bacterial UT[17−20] and *Hp*UreI (2.3 ± 0.14 mM for UT vs ~150 mM for *Hp*UreI), indicating that UT has one or more high-affinity sites for urea. On the other hand, obtaining Hill coefficients of >1 may indicate that *Hp*UreI, like UT,[17−20] has more than one urea binding site(s). In fact, it is tempting to speculate whether the Hill coefficients of 2 and 2.7 [at pH 7.5 and 5.0, respectively (Figure 8)] could be indicative of a different number of urea binding sites in *Hp*UreI. Note that the 35% increase in the level of binding of [^{14}C]urea at pH 5.0 corresponds to the higher Hill coefficient. Further functional and structural studies of *Hp*UreI will shed light on how these channels conduct solutes and determine the similarities and differences between UT and *Hp*UreI transport mechanisms.

Mechanism of Acid Acclimation by *H. pylori*. Both *Hp*UreI and urease are essential for *H. pylori* to resist the acidic environment of the stomach. At acidic pH values, *Hp*UreI conducts urea from the periplasm into the cytoplasm. There, cytoplasmic ureases convert urea into NH_3 and CO_2, which return to the periplasm to neutralize the acid.[8] It has been shown that *Hp*UreI associates with urease in a pH-dependent manner, and that the products of urease activity (NH_3 and CO_2) are able to diffuse through *Hp*UreI.[36]

The accumulation of high levels of NH_3 inside the cell causes alkalization of the cytoplasm and can be toxic for bacteria. Therefore, the cytoplasm should be protected from the products of urea hydrolysis. It has been suggested that the association of urease and *Hp*UreI prevents the dissipation of the products of urease activity into the cytoplasm.[28,29,36] The trimeric association of urease and *Hp*UreI should seal the active site of urease to prevent the escape of the products into the cytoplasm. The active site should also be impervious to water; otherwise, the generated NH_3 would change the cytoplasmic pH by interacting with bulk water and becoming NH_4^+. Because water molecules can diffuse through *Hp*UreI (based on our results), the water molecules necessary for the enzymatic reaction most likely diffuse from the periplasm to the active site through *Hp*UreI. On the basis of our results and previous studies, we propose the following mechanism for acid acclimation by *H. pylori* (Figure 9). Under acidic conditions, *Hp*UreI conducts urea and water into the active site of urease where they are converted into NH_3 and CO_2. The products are then transferred to the periplasm (their final destination) by *Hp*UreI. In this mechanism, the cytoplasm is completely protected from the substrate and products of the reaction. Further studies are necessary to determine the detailed mechanism of action of the *Hp*UreI–urease complex.

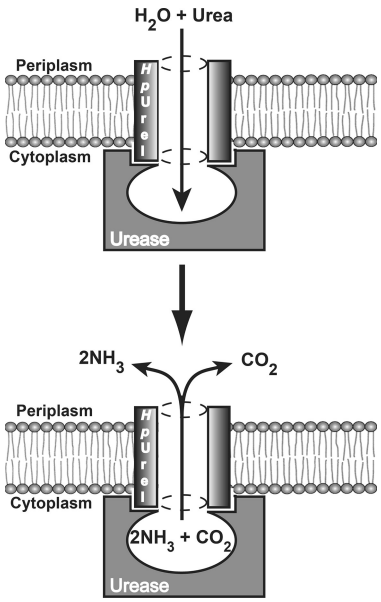

Figure 9. Mechanism of acid acclimation by *H. pylori*. The association of trimeric urease with trimeric *Hp*UreI connects the active site of urease directly to the periplasm through the urea channel. Under acidic conditions, urea and water reach the active site of urease through the urea channel. The products of the urease activity (NH_3 and CO_2) are also released into the periplasm through *Hp*UreI. The association of the urease–*Hp*UreI complex prevents the leakage of potentially toxic products into the cytoplasm.

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ABBREVIATIONS

SPA, scintillation proximity assay; UT, urea transporter; DM, decyl β-D-maltoside; DTT, dithiothreitol; β-ME, β-mercaptoethanol; apparent $K_{0.5}$, half-saturation concentration for urea permeation by *Hp*UreI; IC_{50}, concentration of half-maximal inhibition.

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