Published in final edited form as:

Biochemistry. 2011 October 11; 50(40): 8656–8663. doi:10.1021/bi200887a.

The transport kinetics and selectivity of *HpUreI*, 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, *HpUreI*. Hence, *HpUreI* 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 have developed conditions for the high-yield expression and purification of stable recombinant *HpUreI* that allowed its detailed kinetic characterization in solubilized form and reconstituted into liposomes. Detergent-solubilized *HpUreI* forms homo-trimer, as determined by chemical cross-linking. Urea dissociation kinetics of purified *HpUreI* were determined by means of the scintillation proximity assay (SPA), whereas urea efflux was measured in *HpUreI*-containing proteoliposomes using stopped-flow spectrometry to determine the kinetics and selectivity of the urea channel. The kinetic analyses revealed that urea conduction in *HpUreI* is pH sensitive and saturable with a half-saturation concentration (or $K_{0.5}$) of ~163 mM. Binding of urea by *HpUreI* 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 *HpUreI* is highly selective for urea and hydroxyurea. Removing either amino group of urea molecules diminishes their permeability through *HpUreI*. Similar to urea conduction, water diffusion through *HpUreI* is pH-dependent with low water permeability at neutral pH.

Keywords

urea channel; proteoliposomes; *HpUreI*; solute permeability; urea transport

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*

[†]The authors thank University of Iowa, Carver College of Medicine for funding.

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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 for its colonization of the acidic stomach (3–6). Whereas *H. pylori* is a neutralophilic bacterium which grows optimally at pH 7.0, in the presence of urea it can grow below pH 4.0 (7).

H. pylori uses a unique mechanism to take advantage of urea for neutralizing the acidic stomach (8, 9). Urea is taken up from the stomach environment via an inner membrane urea channel (*HpUreI*). 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 the activity of a membrane-associated carbonic anhydrase, providing buffering in the range of pH 6.1 (Fig. 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). *HpUreI* is essential for *H. pylori* to neutralize the acid and survive in the stomach (8, 10, 12, 13).

HpUreI 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*. 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 due to cytoplasmic alkalization. With a molecular mass of 21.7 kDa (195 amino acids), *HpUreI* is one of the smallest pH-gated channels known.

There are multiple urea transport systems facilitating urea permeation across cell membranes including, but not limited to, urea transporters (UT) (15, 16) and acid-sensitive urea channels (UreI) (8). UT and *HpUreI* are very different in matter of sequence and topology. Unlike *HpUreI*, which has 6 transmembrane domains, UT consists of 12 transmembrane domains (17). Recent functional and structural studies of UT family members have indicated that they follow a channel-like mechanism (17–20). Compared to UT, our knowledge of the structure and mechanism of *HpUreI* channels is very limited.

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

Materials and Methods

Expression and purification of HpUreI

The genomic DNA of *Helicobacter pylori* strain 26695 (purchased from American Type Culture Collection, ATCC) was used as the template for the PCR amplification of the *ureI* gene. The amplified fragment was digested with suitable restriction enzymes and ligated into pET29b vector, which had been digested with the same restriction enzymes. In order to remove His tag during protein purification, a TEV cleavage site (ENLYFQG) was engineered between the *ureI* gene and a His tag.

The expression vector was transformed into *E. 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). Optimum expression was achieved by inducing cell cultures with 0.1 mM IPTG at an OD₆₀₀ 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 xg for 1 hour. 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 βME) at 4 °C. The solubilized protein was loaded onto a Ni²⁺ 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 βME). The resin was washed first with 20 mM and then 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 residual undigested proteins were removed by passing over a Ni²⁺ 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, 4mM DM and 2 mM dithiothreitol (DTT). The symmetric peak of *HpUreI* was collected and its purity was confirmed by SDS-PAGE. The size-homogeneity of the purified protein was routinely confirmed by re-injection into the size-exclusion chromatography column. All chemicals and buffers were purchased from Sigma.

Cross-linking experiments

Glutaraldehyde was used to cross-link *HpUreI* monomers. For the cross-linking reaction, *HpUreI* was purified in phosphate buffer (20 mM KPi pH 7.4, 200 mM NaCl, 10% glycerol, and 4 mM DM). To initiate the cross-linking reaction glutaraldehyde was added to the final concentration of 100mM. To monitor the progress of the reaction, aliquots (25μl) were removed from the reaction mixture at various time points of 1, 2.5, 5, 20, 60, 120 min, and the reaction was quenched by the addition of 1M Tris, pH 7.5 (final concentration of 142mM). The samples were analyzed on a 4–12% gradient SDS-PAGE by silver staining.

Proteoliposomes reconstitutions

E. coli polar lipids (purchased as a chloroform solution from Avanti Polar Lipids, Inc.) were dried under N₂, washed twice with pentane, and further dried by speedvac for 1 hour. The dried lipid was resuspended in 20 mM HEPES pH 7.5, 200 mM NaCl, 2 mM DTT to 10 mg/ml. The lipid suspension was sonicated to clarity, and diluted with a DM buffer (~ 7 mM DM, 20 mM HEPES pH 7.5, 200 mM NaCl, 2 mM DTT) to 2.5 mg/ml lipid and incubated for 30 minutes at room temperature. Protein was added to a final lipid to protein weight ratio of 100 (Xmg protein to Xmg lipid), and incubated for 1 hour at room temperature. Detergent was removed by incubation with Bio-beads SM-2 (Bio-Rad). The vesicles were extruded (through a 0.4 µm filter) multiple times for homogeneity. The vesicles were harvested by centrifugation at 80000xg for 1 hour 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 Na Acetate pH 5.0. To determine the pH profile of *HpUreI*, the vesicles were prepared at the pH range of 4.0 to 7.5 using a 20 mM citrate-phosphate buffer adjusted to the various pH values.

Solutes 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 *HpUreI* the permeability assays were carried out at pH 5.0, where the channel is conductive. Each solute was loaded into the vesicles (control liposomes or *HpUreI* proteoliposomes) by overnight incubation in assay buffer (20mM Na 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 make an isoosmotic solution. The osmolality of each buffer was determined using a Vapro vapor pressure osmometer (Wescor, Inc.). Upon mixing, the external concentration of the permeant solute is reduced by half generating a solute concentration gradient which drives solute efflux. Solute efflux is followed by water efflux leading to the reduction of the vesicle volume which causes an increase in the intensity of scattered light measured at $\lambda = 440\text{nm}$ (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 5–8 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 3–5 independent permeability measurements.

pH-activity profile of *HpUreI*

The pH profile of *HpUreI* activity was determined from 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 3–5 experiments. Urea permeability of control liposome was also measured at each pH, to determine the background permeabilities.

Measurement of apparent $K_{0.5}$ of HpUreI

To measure the kinetics parameters for urea conduction by HpUreI, the urea flux was measured at various concentration gradients of urea. To that end, HpUreI proteoliposomes were loaded with various concentrations of urea, ranging from 50 mM to 800 mM, by overnight incubation in assay buffer (20 mM citrate-phosphate pH 5.0, 100 mM NaCl) containing various concentrations of urea. The vesicles suspension was rapidly mixed (1:1) with assay solution containing isoosmotic 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 HpUreI 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 drives water efflux and vesicle shrinkage was measured as an increase in the intensity of scattered light at $\lambda = 440$ nm. Typically 5–8 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 3–5 permeability measurements from independent proteoliposome preparations. Osmotic water permeabilities were calculated from the measured rate constants using Mathai and Zeidel approaches (23).

HpUreI scintillation proximity binding assay

Binding of 181 μ M 14 C-urea (55 mCi/mmol; American Radiolabeled Chemicals, Inc.) to recombinant HpUreI was assayed by means of the scintillation proximity assay (SPA) (17, 24, 25). Cu^{2+} -coated YSi SPA beads (Perkin Elmer # RPNQ00096) were diluted to 2.5 mg/mL in 150 mM Tris/Mes, pH 7.5 or pH 5.0/50 mM NaCl/20% glycerol/1 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma Co)/0.1% n-dodecyl- β -D-maltopyranoside. 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 non-labelled urea to individual wells of clear-bottom/white-wall 96-well plates (Greiner #655095). Plates were incubated in the dark at 4 °C with vigorous shaking on a vibrating platform for 15 minutes before they were counted in the SPA mode of a Wallac 1450 MicroBetaTM plate PMT counter. Non-specific 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 or cpm) to obtain the UreI-specific binding activity. Data points represent the mean $\pm$ standard error of triplicate determinations. Data fits of kinetic analyses were performed using non-linear regression algorithms in Prism and errors represent the S.E.M. of the fit.

Results and Discussion

Cloning, expression and purification of HpUreI

Several constructs of the *ureI* gene in different pET vectors, including pET29b, pET28b and pET27b, were built and screened in order to identify the best conditions for over-expression

of *HpUreI*. In addition, several growth and expression variables were screened to determine the best conditions of *HpUreI* overexpression. The yield was estimated to be ~0.5 mg protein per Liter of media.

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

The DM extracted *HpUreI* 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) were removed by a second Ni^{2+} affinity column. The TEV digested *HpUreI* was further purified by size-exclusion chromatography (Fig. 2A). *HpUreI* eluted from Superose 6 10/300 GL column as a single peak with a retention volume of 16.3 ml corresponding to a molecular mass of less than 150 kDa. SDS-PAGE analysis confirmed the purity of the protein (Fig 2B, lane 1).

Oligomeric state of detergent-solubilized *HpUreI*

To determine the oligomeric state of detergent-solubilized *HpUreI* 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 4–12% gradient SDS-PAGE (Fig. 2B, lanes 2–7). As the reaction progressed the ~20 kDa band (representing the monomer form of *HpUreI*) disappeared and two bands emerged migrating at ~40 kDa and ~60 kDa which represent the dimer and trimer forms of the protein, respectively. Bands larger than trimer were not observed even after 24 hours exposure to the cross-linker. The glutaraldehyde cross-linking analysis indicates that detergent-solubilized *HpUreI* exists as homo-trimer.

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 be the active form of *HpUreI* in cell membrane.

The results of both co-immunoprecipitation analysis (28) and immunoelectron microscopy experiments (29) showed that cytoplasmic urease interacts with *HpUreI*. The functional unit of *H. pylori* urease includes two different subunits (α and β) (30). The active urease from *H. pylori* consist of three ($\alpha\beta$) heterodimers forming a trimeric assembly ($\alpha\beta$)₃, although the crystal packing of *H. pylori* urease shows a dodecameric assembly of [($\alpha\beta$)₃]₄ (31). Based on our results, one can conclude that *HpUreI* 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 which 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. *HpUreI*-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 isoosmotic 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 $\lambda=440\text{nm}$, as a function of the rate of urea efflux (Fig. 3). Loading vesicles with self-quenching concentrations of carboxyfluorescein is another common method to monitor 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 $(0.032 (\pm 0.007) \times 10^{-6} \text{ cm/s}$ and $0.033 (\pm 0.008) \times 10^{-6} \text{ cm/s}$, respectively) (Fig. 3; green and red traces). The urea permeability coefficient of *HpUreI* proteoliposomes at pH 7.5 is $0.058 (\pm 0.013) \times 10^{-6} \text{ cm/s}$, which is very close to that of control liposomes at either pH (Fig. 3; blue trace). However, *HpUreI* proteoliposomes at pH 5.0 have a urea permeability coefficient of $21.0 (\pm 0.83) \times 10^{-6} \text{ cm/s}$ (Fig. 3; black trace), indicating significant increase (>600-fold) with respect to the control liposome permeability.

pH-activity profile of *HpUreI*

Using the oocyte expression system, *HpUreI* was shown to be a pH sensitive channel with highest activity at acidic pH (8, 35). Our stopped-flow assays also show that *HpUreI* displays a significant increase in urea conduction (more than 350-fold) when the pH decreases from 7.5 to 5.0 $(0.058 (\pm 0.013) \times 10^{-6} \text{ cm/s}$ vs. $21.0 (\pm 0.83) \times 10^{-6} \text{ cm/s}$) (Fig. 4). Previous results have shown that urea uptake in *HpUreI*-expressing *Xenopus* oocytes at pH 5.0 was only 6- to 10-fold higher than that in either injected oocytes at pH 7.5 or non-injected oocytes at either pH (8). The lower activities observed in *Xenopus* oocyte expression may be due to the instability of prokaryotic *HpUreI* in this eukaryotic expression system. However, previous *in vivo* assays (using oocytes) (35) and our *in vitro* pH profile assay show that decreasing pH activates urea conduction by *HpUreI* and that the pH of the half-maximal activation is ~ 5.9 ($\text{pH}_{0.5}=5.9$, corresponding to a H^+ concentration of $1.26 \mu\text{M}$) (Fig. 4). The periplasmic domains are suggested to be involved in the pH gating of *HpUreI* (14). The $\text{pH}_{0.5}$ 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 *HpUreI*.

Kinetics measurements of urea conduction by *HpUreI*

The stopped-flow proteoliposome assay was used to determine the kinetics of urea conduction by *HpUreI*. Proteoliposomes containing *HpUreI* were loaded with various urea concentrations and the efflux of urea was measured at pH 5.0 under isoosmotic conditions (Fig. 5). The data were fitted to the Michaelis-Menten equation and the half-saturation concentration for urea permeation by *HpUreI* (apparent $K_{0.5}$) was determined to be $163 \pm 20 \text{ mM}$. This apparent $K_{0.5}$ for urea conduction in *HpUreI* is very close to previously reported values for UTs (218 mM for mammalian erythrocyte UT-B (18), 182 mM (19) or $104 \pm 9.96 \text{ mM}$ (20) for bacterial *ApUT*). It seems, despite their differences in the sequence and

topology, UT and UreI families may share similar mechanism of solute conduction. Consistent with our results, Weeks *et al.* showed that a concentration gradient up to 100mM does not saturate the solute conduction (8). We observed that the saturation of the solute conduction occurs only when urea concentration gradient is above 400–500 mM.

Solute selectivity of the channel

Solute selectivity is one of the most distinguishing features of a channel protein. Aside from urea, which molecules can pass through urea channels has not been thoroughly investigated. Stopped-flow light-scattering measurements were used to investigate the conduction of various urea analogues through *HpUreI*. 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 similar manner as urea.

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

Thiourea (the =O of urea was replaced by =S) diffuses through *HpUreI* with a ~62% decrease in 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) (Fig. 6). Contradictory to a previous report suggesting that *HpUreI* does not conduct thiourea (8), our results indicate that *HpUreI* 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), –CH₃ (acetamide) or –CH₂CH₃ (propionamide). These solutes diffuse through lipid bilayer, and consequently display permeability through control liposomes. Our results show that the permeability of these three solutes through *HpUreI* proteoliposomes is not significantly higher than that of control liposomes (Fig. 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 *HpUreI*.

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 permeability of methyl- and ethylurea are 4-fold less than that of urea (Fig. 6). Overall, our results indicate that the urea channel is highly selective for urea and hydroxyurea. Moreover, the solute selectivity of *HpUreI* resembles that of UT. These results imply similarity in the solute pathways of UTs and *HpUreI* channels.

Water permeability through *HpUreI* proteoliposomes were 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 based on light scattering. At pH 5.0, the osmotic water permeability coefficient of *HpUreI* proteoliposomes displayed a 6.6 fold increase compared to control vesicles ($52.1 (\pm 2.1) \times 10^{-6}$ cm/s vs. $8.0 (\pm 1.9) \times 10^{-6}$ cm/s), indicating that water diffuses

through the channel (Fig. 7). The osmotic water permeability coefficient of *HpUreI* at pH 7.5 was ~2.5-fold higher than that of control liposomes ($18.4 (\pm 1.3) \times 10^{-6}$ cm/s vs. $7.5 (\pm 2.2) \times 10^{-6}$ 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 *HpUreI* is pH-sensitive and suggests that water and urea share a common conduction pathway.

The binding affinity of urea to *HpUreI*

Binding kinetics of *HpUreI* for urea was measured at pH 5.0 and 7.5 in order to determine how the changes in pH affect urea binding to the channel. The copper chelate affinity-based scintillation proximity assay (SPA), was used to directly measure the binding of C^{14} -urea to the detergent-solubilized *HpUreI* channel. The SPA assay has been used previously to measure the affinity of various ligands to their cognate transporters and channels, even when the affinity is relatively low (17, 24). Competition of binding of $181 \mu\text{M } C^{14}$ -urea with non-labeled urea revealed half-maximum inhibition constants (IC_{50}) of 153 ± 34 mM and 149 ± 23 mM at pH 5.0 and pH 7.5, respectively (Fig. 8). This apparent affinity for urea binding is consistent with the apparent $K_{0.5}$ value for urea conduction determined using the proteoliposome assay ($K_{0.5} = 163 \pm 20$ mM). Notably, however, whereas the apparent urea affinity is independent of the pH, binding of $181 \mu\text{M } C^{14}$ -urea at pH 5.0 was about 35 % higher than that observed at pH 7.5. In addition, fitting the data to the Hill equation revealed a Hill coefficient (a measure for 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 *HpUreI* (2.3 ± 0.14 mM for UT vs. ~150 mM for *HpUreI*), indicating that UT has one or more high affinity sites for urea. On the other hand, obtaining Hill coefficients larger than 1 may indicate that *HpUreI*, 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, Fig. 8) could be indicative of a different number of urea binding sites in *HpUreI*. Note that the 35 % increase in the binding of ^{14}C -urea at pH 5.0 corresponds to the higher Hill coefficient. Further functional and structural studies of *HpUreI* will shed light on how these channels conduct solutes and determine the similarities and differences between UTs and *HpUreI* transport mechanisms.

The mechanism for acid acclimation by *H. pylori*

Both *HpUreI* and urease are essential for *H. pylori* to resist the acidic environment of the stomach. At acidic pH values *HpUreI* undergoes a conformation change which allows for urea conduction and conducts urea from the periplasm into the cytoplasm. There cytoplasmic ureases convert urea into NH_3 and CO_2 , which return into the periplasm to neutralize the acid (8). It has been shown that *HpUreI* 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 *HpUreI* (36).

The accumulation of high levels of NH_3 inside the cell causes alkalization of the cytoplasm and can be toxic for bacterium. Therefore, the cytoplasm should be protected from the

products of urea hydrolysis. It has been suggested that the association of urease and *HpUreI* prevents the dissipation of the products of urease activity into the cytoplasm (28, 29, 36). The urease-*HpUreI* trimer-trimer complex should seal the active site of urease in order 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^+ . Since water molecules can diffuse through *HpUreI* (based on our results), the water molecules necessary for the enzymatic reaction most likely diffuse from the periplasm to the active site through *HpUreI*. Based on our results and previous studies we propose the following mechanism for acid acclimation by *H. pylori* (Fig. 9). Under acidic conditions, *HpUreI* 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 *HpUreI*. In this mechanism the cytoplasm is completely protected from the substrate and products. Further studies are necessary to determine the mechanism of action for *HpUreI*-urease complex.

Acknowledgments

The authors thank University of Iowa, Carver College of Medicine for funding.

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Figure 1.

Figure 2A

Figure 2B

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.