Mechanism of Proton Gating of a Urea Channel*

David L. Weeks, Gene Gushansky, David R. Scott, and George Sachs‡

From the Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California 90073 and University of California, Los Angeles, California 90024

The size and complexity of many pH-gated channels have frustrated the development of specific structural models. The small acid-activated six-membrane segment urea channel of Helicobacter hepaticus (HhUreI), homologous to the essential UreI of the gastric pathogen Helicobacter pylori, enables identification of all the periplasmic sites of proton gating by site-directed mutagenesis. Exposure to external acidity enhances $[^{14}C]u$rea uptake by Xenopus oocytes expressing HhUreI, with half-maximal activity ($p_{\text{H}}_{0.5}$) at pH 6.8. A downward shift of $p_{\text{H}}_{0.5}$ in single site mutants identified four of six protonatable periplasmic residues (His-50 at the boundary of the second transmembrane segment TM2, Glu-56 in the first periplasmic loop, Asp-59 at the boundary of TM3, and His-170 at the boundary of TM6) that affect proton gating. Asp-59 was the only site at which a protonatable residue appeared to be essential for pH gating. Mutation of Glu-110 or Glu-114 in PL2 did not affect the $p_{\text{H}}_{0.5}$ of gating. A chimera, where the entire periplasmic domain of HhUreI was fused to the membrane domain of Streptococcus salivarius UreI (SsUreI), retained the pH-independent properties of SsUreI. Hence, proton gating of HhUreI likely depends upon the formation of hydrogen bonds by periplasmic residues that in turn produce conformational changes of the transmembrane domain. Further studies on HhUreI may facilitate understanding of other physiologically important pH-responsive channels.

The $ureI$ genes found in gastric and oral bacteria such as Helicobacter pylori, Streptococcus salivarius (HpUreI and SsUreI), and most recently in an enteric bacterium, Helicobacter hepaticus (HhUreI), encode a family of inner membrane urea channels (1–3). This family has homology to the six-transmembrane segment putative amide bacterial transporters but has none with the 10-transmembrane segment-containing family of urea channels present in multiple species of bacteria and mammals (4–6). HpUreI has been shown previously to be acid-activated, alternating between open and closed conformations with only extracellular protons as activating ligands (7–10). Expression of UreI is essential for survival of H. pylori in acid at physiological urea concentrations in vitro and for infection of either mouse or gerbil stomach (11–12). HpUreI accelerates urea flux across the inner membrane and thereby increases access of medium urea to the intrabacterial urease by 300-fold as compared with uptake by simple diffusion (9). The increased rate of uptake under acidic conditions results in a 10- to 20-fold increase of intrabacterial urease activity increasing buffering of cytoplasmic and periplasmic pH (8). Channel closure at neutral pH serves to safeguard against lethal alkalization (13) due to excessive urease activity.

Among the UreIs, the six transmembrane segments are ~85% homologous, and the cytoplasmic domain is also conserved; however, the periplasmic domains, with either 24 (SsUreI and HhUreI) or 47 (HpUreI) amino acids, are non-conserved. HpUreI is acid-activated, whereas SsUreI is pH-independent. SsUreI has only one protonatable residue in its periplasmic domain in comparison to the fourteen present in HhUreI. The paucity of protonatable residues in the former is consistent with the pH independence of this urea channel (9).

Here we show that UreI of H. hepaticus, with only 24 periplasmic amino acids, is proton-gated like HpUreI. Being composed of only 170 amino acids, HhUreI represents the simplest known acid-gated channel. In contrast to mutations of HpUreI (9, 10, 14), active mutants of all six protonatable periplasmic amino acids of HhUreI could be generated with almost full retention of urea transport at acidic pH, allowing analysis of each of their roles in pH gating. While it was possible to observe acid activation in histidine-less mutants, both histidines and carboxylic acid residues likely coordinate to create a distributed pH sensor with a $p_{\text{H}}_{0.5}$ of 6.8, nearly one pH unit higher than in HpUreI. Further, the pH independence of chimeras containing the SsUreI membrane domain and the HhUreI periplasmic domain indicates that protonation-induced periplasmic conformational changes must also affect the membrane conformation to produce acid gating in HhUreI.

**EXPERIMENTAL PROCEDURES**

Urease Assays—The pH8080 (15) plasmid encoding the entire H. pylori urease gene cluster and NixA was mutated using the QuikChange mutagenesis system (Stratagene) to create the E135D mutation in ureI shown previously to completely inactivate channel activity of HpUreI. The resulting plasmid pH8080/E135D was complemented in trans by pBlSK (Stratagene) containing ureI of H. pylori or H. hepaticus. SES5000 Escherichia coli containing both pH8080/E135D and pBlSK/UreI were grown overnight in Luria Broth (LB) supplemented with 20 μM NiCl$_2$, 100 μM of overnight culture was added to 900 μl of Hp Buffer (100 mM NaH$_2$PO$_4$, 138 mM NaCl, 0.5 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM glucose, 1 mM glutamine, and 5 mM $[^{14}C]$urea with a specific activity of 10 mCi/mmol) at various pHs and incubated for 30 min at 37 °C with constant agitation. Urease activity was measured radiometrically (16) where plastic wells containing 0.5 mM KOH-soaked filter paper hung from rubber stoppers were used to collect the total $^{14}$CO$_2$ that resulted from the hydrolysis of urea by urease. The reaction mixture was terminated by the addition of 5 v/v H$_2$SO$_4$ to expel all $^{14}$CO$_2$ from solution. The wells were placed in scintillation mixture (Hionic-Flour, Packard Instruments, Meriden, CT), and the radioactivity was measured...
measured by scintillation counting. Urease activity is reported as nml of CO2 released/min/mg of protein. Protein concentration was determined using the Pierce protein assay. There was no change of medium pH during the incubation. Data represent three independent assays performed in triplicate.

Oocyte Vectors and Mutagenesis—Point mutants and chimeras of the ureI genes were constructed using a two-step method of site-directed mutagenesis (9). Mutagenic PCR products were digested with XbaI and KpnI (New England Biolabs) and ligated into pcDNA3.1—(Invitrogen) downstream of a T7 promoter and upstream of a 170-bp poly(A) tract. The poly(A) tract, not present in the parental vector used previously, increased protein expression ~5-fold over levels reported in previous studies (7, 9). To achieve comparable steady state levels of uptake and to avoid premature equilibration, the time of incubation was shortened to 6 min from the previous time of 30 min. As a result of shorter reaction times, background urea uptake due to UreI-independent diffusion of urea across the oocyte plasma membrane was significantly reduced. All constructs were sequenced (Keck Sequencing Facility, New Haven, CT) prior to use as a template for RNA generation.

RNA Preparation—10 μg of ureI pcDNA3.1— vectors were linearized by digestion with EcoRI (New England Biolabs). The reaction product was column-purified (Wizard preps, Promega) and eluted in 50 μl to achieve a final concentration of 200 ng/μl. The linearized vectors were then used as templates for in vitro transcription to capped RNA using the mMessage mMachine cRNA kit (Ambion) according to the manufacturer’s recommendations.

Urease Uptake Assays—Xenopus laevis oocytes were injected with 46.6 nl of cRNA (1 μg/μl) using the Nanobip II injection system (Drummond) at room temperature and then incubated at 18 °C in Barth’s solution (88 mM NaCl, 0.82 mM MgSO4, 0.41 mM CaCl2, 1 mM KCl, 0.33 mM Ca(NO3)2, 2.4 mM NaHCO3 and 10 mM Hepes, pH 7.4) for 24 h to allow for expression prior to performing the assay. 6–8 oocytes containing each cRNA were transferred into 1 ml of Ringer’s reaction buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 50 μM [14C]urea buffered with 20 mM Mes (pH 6.0) or 20 mM Hepes (pH 6.5)) at room temperature for 6 min. The oocytes were then washed twice by transfer to 25 ml of Barth’s solution (pH 7.4) to remove any external [14C]urea and then transferred to individual vials for quantification by liquid scintillation counting. For measurement of pH dependence of wild-type H. pylori ureI in Xenopus oocytes was pH-dependent with half-maximal urea uptake at pH 6.8 (Fig. 1b). This is defined here as the pH0.5 of wild-type H. pylori ureI. Background urea uptake in mock-injected oocytes was less than 1 pmol of urea/oocyte/6 min (Table I), whereas H. pylori ureI-injected oocytes had uptake of ~11 pmol of urea/oocyte/6 min at pH 5.0, a 19-fold enhancement of urea transport. Urea uptake at pH 7.5 was also greater in oocytes injected with wild-type H. pylori ureI than when injected with inactive H. pylori ureI mutants or in sham-injected oocytes. Hence, wild-type H. pylori ureI has a low but finite open probability at neutral pH. H. pylori ureI has a lower pH0.5 of activation of 6.0 (9), and unlike H. pylori ureI, uptake by H. pylori ureI is undetectable at neutral pH (Table I). SaUreI-catalyzed urea uptake was pH-independent, and the maximal rate of urea uptake was slightly higher than for H. pylori ureI-expressing oocytes (Fig. 1b).

Effects of Site-directed Mutagenesis of H. pylori ureI

Fig. 2 shows a model of the two-dimensional structure of the H. pylori ureI accompanied by the alignment of H. pylori ureI and SaUreI. The putative periplasmic domains, PL1, PL2, and the C terminus, are relatively well defined by hydrophilic amino acids at their boundaries and by various predictive algorithms. All six protonatable residues and two asparagines in the H. pylori periplasmic domain, the glutamic acid conserved in the sixth transmembrane segment (TM6) of all UreIs, and the lone histidine in the cytoplasmic domain of H. pylori ureI were mutated to identify protonatable residues relevant to pH activation.

Replacement of the single histidine, His-23, in the cytoplasmic domain with Asn or replacement of the lone glutamic acid in the membrane domain by glutamine had no effect on urea transport. Hence, these residues are not implicated in medium pH regulation of urea transport (data not shown).

RESULTS

Effects of Medium pH on Wild-type H. hepaticus UreI

Urease Activity in Transformed E. coli—Urease activity was measured as a function of medium pH in intact E. coli expressing active H. pylori ureI and an inactivated HpUreI as detailed under “Experimental Procedures.” Complementation with wild-type H. pylori ureI or HpUreI then allowed a 6-fold acid activation of urease with half-maximal activity at pH 7.1 and 6.2, respectively, due to urea uptake through UreI (Fig. 1a).

Although useful, this method of assessing UreI channel gating is an indirect one sensitive to levels of urease expression. The pH of half-maximal activation of various constructs obtained using this system was right-shifted by 0.2–0.3 units as compared with direct measurements of activation obtained via quantification of uptake into Xenopus oocytes. The shift in pH0.5 was most likely the result of limited urease expression at pHs above those required to achieve maximal channel open probability.

pH Activation of Transport by the UreIs in Xenopus Oocytes—The uptake of [14C]urea into oocytes expressing the wild-type H. pylori ureI in Xenopus oocytes was pH-dependent with half-maximal urea uptake at pH 6.8 (Fig. 1b). This is defined here as the pH0.5 of wild-type H. pylori ureI. Background urea uptake in mock-injected oocytes was less than 1 pmol of urea/oocyte/6 min (Table I), whereas H. pylori ureI-injected oocytes had uptake of ~11 pmol of urea/oocyte/6 min at pH 5.0, a 19-fold enhancement of urea transport. Urea uptake at pH 7.5 was also greater in oocytes injected with wild-type H. pylori ureI than when injected with inactive H. pylori ureI mutants or in sham-injected oocytes. Hence, wild-type H. pylori ureI has a low but finite open probability at neutral pH. H. pylori ureI has a lower pH0.5 of activation of 6.0 (9), and unlike H. pylori ureI, uptake by H. pylori ureI is undetectable at neutral pH (Table I). SaUreI-catalyzed urea uptake was pH-independent, and the maximal rate of urea uptake was slightly higher than for H. pylori ureI-expressing oocytes (Fig. 1b).

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Histidine Mutations in the Periplasmic Domain

Mutation of His-50 in PL1 to either glutamine or leucine resulted in an increase of urea uptake at pH 7.5 with near wild-type uptake at pH 5.0 (Table I and Fig. 3). Hence, there was retention of acid activation but a decreased stability of the closed conformation at alkaline pH. The pH0.5 of H50L was left-shifted to pH 6.3 as compared with the wild-type pH0.5 of 6.8. The pH0.5 of H50Q was decreased slightly farther to pH 6.2. Mutation of His-50 to the positively charged Lys resulted in a fully active channel at both neutral and acidic pH. Other mutations such as H50Y and H50N showed less than 30% of wild-type maximal activity and were not further studied (data not shown).

 Mutation of the C-terminal His-170 to Gln or Lys lowered the pH0.5 to 6.3 suggesting that this histidine may also be involved in proton gating of wild-type HhUreI (Table I and Fig. 3).

### Table I

| Urea Transport | pH 5.0 | pH 7.5 | pH0.5 |
|----------------|-------|-------|-------|
| Wild-type UreI | 11.4 ± 0.4 | 1.3 ± 0.1 | 6.8 |
| S. salivarius UreI | 14.5 ± 0.2 | 14.3 ± 0.2 | 6.2 |
| H. pylori UreI | 13.0 ± 0.4 | 0.7 ± 0.1 | 6.0 |
| Sham-injected | 0.6 ± 0.1 | 0.5 ± 0.1 | n.d. |

#### Mutations of H. hepaticus UreI

| Mutation | pH 5.0 | pH 7.5 | pH0.5 |
|----------|-------|-------|-------|
| H23N     | 15.4 ± 0.6 | 1.8 ± 0.7 | n.d. |
| H50Q     | 10.4 ± 0.6 | 4.1 ± 0.5 | 6.3 |
| H50L     | 13.2 ± 0.6 | 3.8 ± 0.6 | 6.3 |
| H50K     | 10.7 ± 0.3 | 9.5 ± 0.3 | 6.8 |
| N54T     | 12.8 ± 0.4 | 1.7 ± 0.3 | 6.8 |
| E56Q     | 9.7 ± 0.4 | 0.8 ± 0.1 | 6.2 |
| N58T     | 11.9 ± 0.2 | 1.1 ± 0.1 | 6.4 |
| D59E     | 8.8 ± 0.3 | 1.5 ± 0.1 | 6.1 |
| D59N     | 1.3 ± 0.1 | 0.4 ± 0.1 | n.d. |
| E110Q    | 12.6 ± 0.6 | 1.8 ± 0.3 | 6.7 |
| E114Q    | 11.6 ± 0.8 | 3.5 ± 0.4 | 7.0 |
| E114D    | 2.7 ± 0.4 | 0.9 ± 0.2 | n.d. |
| H170K    | 12.4 ± 0.8 | 1.3 ± 0.1 | 6.3 |
| H170Q    | 11.0 ± 1.0 | 1.7 ± 0.6 | 6.3 |
| H50Q/H170Q | 10.4 ± 0.5 | 2.2 ± 0.5 | 6.2 |

#### Mutations of S. salivarius UreI

| Mutation | pH 5.0 | pH 7.5 | pH0.5 |
|----------|-------|-------|-------|
| E56Q     | 12.4 ± 0.4 | 14.3 ± 0.4 | n.d. |

**Fig. 2. Two-dimensional model and alignment of H. hepaticus and S. salivarius UreI.** At the top is shown a two-dimensional model of the Hh- and SsUreIs (GenBankTM/EBI accession numbers AAK69200 and AAC72025) indicating the relative positions of the protonatable amino acids mutated in these studies. Below is shown the alignment of H. hepaticus and S. salivarius UreI with arrows demarcating the position of chimeric substitutions.
Mutation of both periplasmic histidines in HhUreI to glutamine (H50Q/H170Q) had an effect similar to that of the H50Q mutation, namely, increased uptake at alkaline pH and a left-shifted pH0.5 of channel activation (Table I and Fig. 3). Histidines, therefore, are not essential for the acid gating of HhUreI, but their presence close to the transmembrane boundaries of TM2 and TM6 affects the pH profile of activation.

**Carboxylic Amino Acid Mutations in the Periplasmic Domain**

Three glutamates and one aspartate are predicted to be in the HhUreI periplasmic domain (Fig. 2). The participation of these residues in pH sensing is strongly suggested by the observation of pH gating in the histidine-less mutant (H50Q/H170Q). Substitution of Glu-56 by Gln in PL1 shifted the pH0.5 to 6.2 (Table I and Fig. 3). Histidines, therefore, are not essential for the acid gating of HhUreI, but their presence close to the transmembrane boundaries of TM2 and TM6 affects the pH profile of activation.

Mutation of both periplasmic histidines in HhUreI to glutamine (H50Q/H170Q) had an effect similar to that of the H50Q mutation, namely, increased uptake at alkaline pH and a left-shifted pH0.5 of channel activation (Table I and Fig. 3). Histidines, therefore, are not essential for the acid gating of HhUreI, but their presence close to the transmembrane boundaries of TM2 and TM6 affects the pH profile of activation.

**HhUreI and SsUreI Chimeras**

The S. salivarius urea channel is medium pH-independent (Table II and Fig. 3) with no homology in the periplasmic domain to either H. pylori or H. hepaticus UreI but 85% homology in the membrane domain (Fig. 2). Minor similarities exist, namely a permanently charged lysine in place of the protonatable histidine in the HhUreI C terminus and a glutamic acid, Glu-59, in place of the critical Asp-59 residue of HhUreI. The mutation, E59Q, in SsUreI had no effect on transport (Table I). The availability of the highly homologous membrane domain of SsUreI allowed evaluation of the contribution of changes in the membrane domain to the different states of HhUreI. Chimeras exchanging the periplasmic loops and C-
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Previously, mutagenesis of the UreI of H. pylori was performed to identify the sites involved in pH gating. Mutations were analyzed either directly, by quantitative measurement of \(^{14}C\)urea uptake into Xenopus oocytes (9), or indirectly, by measurement of urease activity in H. pylori where ureI was mutated by allelic replacement (10) or in an ureI-deficient strain of H. pylori complemented by various mutated ureIs (14). All methods showed that urea transport through HpUreI is acid-activated. However, with regards to characterization of one key residue, His-123, the results of Bury-Mone et al. (10) were found to contradict the findings obtained using Xenopus oocytes. With the exception of this report by Bury-Mone and colleagues (10), functional descriptions of HpUreI expressed in oocytes and H. pylori have been indistinguishable (17). Further, the urea channel of Yersinia pseudotuberculosis, Yut, has been shown to function similarly both in oocytes and in H. pylori (4). Finally, another laboratory (14), analyzing mutants of H. pylori UreI in H. pylori, confirmed many of the original findings of the oocyte studies including those of His-123. Thus, the hypothesis that the mechanism of pH gating might differ when HpUreI is expressed in oocytes as compared with H. pylori requires additional substantiation.

Here we have shown that HpUreI is an acid-gated urea channel like HhUreI whether expressed in E. coli or in Xenopus oocytes. Acidification opens the channel, and protons are the only activating ligands in these experiments. Since the chimera containing the entire periplasmic domain of HpUreI and the membrane and cytoplasmic domains of SsUreI was pH-insensitive, a change of both periplasmic and membrane conformation in HpUreI must result from protonation of the relevant periplasmic residues. HpUreI is homologous in its membrane and cytoplasmic domains to those of HpUreI and SsUreI but differs markedly in the periplasmic domain. In evolution, it seems likely that an archetypical pH-insensitive UreI, perhaps SsUreI, was modified to attain both flexibility in the transmembrane helices and protonatable amino acids that allowed for pH gating. The evolution of the acid gate was probably an adaptation to increased constitutive expression of urease activity and the danger of high activity at alkaline pH for these neutralophiles (13).

Several ion-conducting channels have been shown to be sensitive to either cytoplasmic or external pH. Examples include the vanilloid (VR1) and N-methyl d-aspartate receptors, the Kv1.1 (ROMK) and Slo1-BK channels, the family of acid-sensing ion channels, and an outwardly rectifying Cl\(^-\) channel found in Sertoli cells (19–24). These channels are typically large multisubunit assemblies sensitive to effects on multiple regions of the protein by modulatory and allosteric regulation.

Studies have revealed various mechanisms by which protons can either enhance or abrogate transport. In the case of the vanilloid receptor, the primary effect of extracellular protons is terminal segments were constructed to determine whether the presence of the periplasmic domain of HhUreI was sufficient to confer the property of pH gating onto the pH-insensitive SsUreI.

Chimeras between the SsUreI Periplasmic Regions and the HhUreI Membrane Domain—Replacement of the PL1 of HhUreI with that of SsUreI (Ss1-Hh) resulted in a transporter fully and equally active at alkaline and acidic pH. This result implies that PL1 imparts a specific conformation to the channel that allows channel closure at neutral pH. Furthermore, the presumed role of PL1 in acid gating of this channel would appear to be dependent on a protonation-induced disruption of this closed conformation. All data pertaining to the chimeras are presented in Table II.

Replacement of PL2 of HhUreI with PL2 of SsUreI (Ss2-Hh) inactivated the channel. Presumably, the structure of PL2 may confer stability to the open conformation of the transmembrane helices, stability otherwise inherent in the pH-independent SsUreI. Replacement of the four C-terminal amino acids of HhUreI with the three C-terminal amino acids of SsUreI (SSC2-Hh) resulted in a transporter with about 30% open probability at acidic pH and a closed state at neutral pH. Hence, changes in the C terminus also affect pH gating of this channel as was also seen with the mutation of His-170. As postulated for PL2, the HhUreI C terminus may confer stability to the open conformation of the channel.

**Table II**

| Chimeras with SsUreI membrane domain | pH 5.0 | pH 7.5 |
|-------------------------------------|--------|--------|
| Wt-Ss                               | 14.3 ± 0.2 | 14.3 ± 0.2 |
| Hh1-Ss                              | 11.5 ± 0.6 | 11.2 ± 0.3 |
| Hh2-Ss                              | 11.9 ± 0.5 | 11.7 ± 0.6 |
| HhC3-Ss                             | 0.8 ± 0.1 | 1.0 ± 0.1 |
| HhC3C4-Ss                           | 10.7 ± 0.4 | 11.9 ± 0.3 |
| Hh1.2-Ss                            | 8.9 ± 0.3 | 10.2 ± 0.4 |
| Hh1.2C10-Ss                         | 12.4 ± 0.3 | 12.4 ± 0.6 |

**Chimeras with HhUreI membrane domain**

| Wt-Hh                               | 11.4 ± 0.4 | 1.3 ± 0.1 |
| Ss1-Hh                              | 10.6 ± 0.6 | 10.9 ± 0.6 |
| Ss2-Hh                              | 1.5 ± 0.4 | 0.9 ± 0.2 |
| SSC2-Hh                             | 3.7 ± 0.4 | 0.8 ± 0.2 |

In multioop chimeras with the S. salivarius UreI membrane domain, replacement of both the first and second periplasmic loop with those of HhUreI (Hh1.2-Ss) generated an active channel at both acidic and alkaline pH, similar to the results where the periplasmic loops were substituted individually (Hh1-Ss and Hh2-Ss). All three periplasmic domains could also be simultaneously replaced by those of HhUreI (Hh1.2C10-Ss), with retention of full pH-independent activity like that of the wild-type SsUreI. Hence, the membrane domain of HhUreI, but not that of SsUreI, is able to close in response to deprotonation of the periplasmic domain. Therefore, the properties of the membrane domain of SsUreI appear to be dominant, and changes of conformation in the periplasmic domain of HhUreI are by themselves unable to affect the conformation of the transmembrane helices of SsUreI.

DISCUSSION

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Injected oocytes were placed in uptake medium for 6 min in the presence of 50 μM \(^{14}C\)urea (±S.E., n = 3 with 5–7 oocytes/experiment). The chimeras are named with the source of the periplasmic domain followed by the membrane domain to which they are fused, e.g. Hh1, 2-Ss UreI denotes the first two periplasmic loops of HhUreI fused with the membrane domain of SsUreI.

The C-terminal amino acids of SsUreI are analogous to those of the wild-type UreI. Without disrupting transport at either pH 5.0 or 7.5, protonation of the C terminus does not affect the open probability of the native SsUreI.

UreI denotes the first two periplasmic loops of UreI. Replacement of both PL1 and PL2 of HhUreI with those of SsUreI (HhSsUreI) resulted in a transporter fully active at alkaline and acidic pH and a closed state at neutral pH. Hence, the membrane domain of HhUreI, but not that of SsUreI, is able to close in response to deprotonation of the periplasmic domain. Therefore, the properties of the membrane domain of SsUreI appear to be dominant, and changes of conformation in the periplasmic domain of HhUreI are by themselves unable to affect the conformation of the transmembrane helices of SsUreI.
to potentiate the binding of capsaicin (19). For the acid-sensing ion channels, extracellular protons enhance the release of Ca2+, enabling ion conduction (25). In the voltage-sensitive ROMK channel (Kir1.1) and the Slol-BK channel, intracellular protons modify local surface potential, thereby shifting the voltage dependence of activation (21, 22). pH-sensitive transport of a neutral solute has also been described in aquaporin 3 (AQP3) in which extracellular protons block transport by competing with glycerol for binding sites within the channel conduction pathway (26).

In a manner more analogous to H. pylori and H. hepaticus UreI, protons directly activate transport in channels such as the CIC-2G channel (27), the N-methyl-D-aspartate receptor (in the presence of saturating concentrations of glutamate) (20), and the KATP channel (28). Mutational analysis of the CIC-2G channel showed that transport could be abolished by substitution of one or more carboxylic amino acids (27). Several residues were spatially coordinated to generate an overall pH50 of activation distinct from the individual pKa values of involved side chains.

Given that neutral residue replacement of five of six protonatable periplasmic residues in HhUreI failed to abrogate pH gating, either the single irreproducible residue, Asp-59, must dominate channel activation as in the case of the KATP channel (28), or multiple residues must contribute toward the formation of a more diffusely organized pH sensor. Findings in the current work are in keeping with the latter hypothesis. Individual mutation of at least four residues in PL1 and the C terminus significantly alters the pH50 of HhUreI suggesting that the transition between the open and closed states is accomplished by the restructuring of a network of hydrogen bonds. Finally, as pH gating persists in the absence of periplasmic histidines and the pH50 of 6.8 in the wild type is well above the predicted pKa of an aspartyl or glutamyl side chain, channel gating likely reflects the response of a coordinated system rather than protonation of a single residue.

The requirement of the membrane domain of HhUreI for pH activation in the chimeric constructs with the StUreI membrane domain also showed the need for interaction between periplasmic and membrane domains. The relative simplicity of this pH-gated neutral solute channel, as compared with the ion channels studied thus far, and the ability to generate active chimeras of a pH-gated and a pH-independent channel have, therefore, provided the basis for a model involving both periplasm and membrane in pH activation of urea transport.

A hypothetical model consistent with the mutational data is shown in Fig. 4. It is postulated that the formation of hydrogen bonds between His-50 and Asp-59 as well as between Glu-56 and His-170 results in an increase in separation between TM2, TM3, and TM6. Relative motion of these and an unspecified transmembrane segment (perhaps TM4 since it has a hydrophilic face) allows entry of urea into a monomeric channel under acidic but not neutral conditions. The decision to draw the functional pore as a monomer is based on the lack of support for functional interaction between subunits in co-expression experiments designed to show dominant negative interactions between wild-type and mutant channels. The four transmembrane helices shown forming the pore are predicted to be the minimum number of helices capable of forming a conduction pathway (18, 20) that furthermore would be predicted to confer the high degree of specificity needed to distinguish between urea and thiourea shown for several members of this family (7, 9).

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