Structure of the proton-gated urea channel from the gastric pathogen *Helicobacter pylori*

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Half the world’s population is chronically infected with *Helicobacter pylori*, causing gastritis, gastric ulcers and an increased incidence of gastric adenocarcinoma2. Its proton-gated inner-membrane urea channel, HpUreI, is essential for survival in the acidic environment of the stomach1. The channel is closed at neutral pH and opens at acidic pH to allow the rapid access of urea to cytoplasmic urease3. Urease produces NH3 and CO2, neutralizing entering protons and thus buffering the periplasm to a pH of roughly 6.1 even in gastric juice at a pH below 2.0. Here we report the structure of HpUreI, revealing six protomers assembled in a hexameric ring surrounding a central bilayer plug of ordered lipids. Each protomer encloses a channel formed by a twisted bundle of six transmembrane helices. The bundle defines a previously unobserved fold comprising a two-helix hairpin motif repeated three times around the central axis of the channel, without the inverted repeat of mammalian-type urea transporters. Both the channel and the protomer interface contain residues conserved in the AmiS/UreI superfamily, suggesting the preservation of channel architecture and oligomeric state in this superfamily. Predominantly aromatic or aliphatic side chains line the entire channel and define two consecutive constriction sites in the middle of the channel. Mutation of Trp 153 in the cytoplasmic constriction site to Ala or Phe decreases the selectivity for urea in comparison with thiourea, suggesting that solute interaction with Trp 153 contributes specificity. The previously unobserved hexameric channel structure described here provides a new model for the permeation of urea and other small amide solutes in prokaryotes and archaea.

Treatment of *H. pylori* infection is becoming less effective as a result of increasing antibiotic resistance, suggesting that a specifically targeted approach to eradicate this organism would be beneficial. Colonization of the acidic mammalian stomach by *H. pylori* depends on the presence of the inner-membrane protein HpUreI, making it a viable clinical target4. HpUreI is a proton-gated urea channel that is closed at pH 7.0 and fully open at pH 5.0, enabling the rapid entry of urea into the bacterium5. Urease activity (Supplementary Fig. 1) buffers the periplasm to pH 6.1, which is essential for the survival of *H. pylori* at acidic pH as a result of the neutralizing capacity of NH3 and the generation of HCO3 by periplasmic α-carboxylic anhydrase. Conversely, the closure of HpUreI at neutral pH prevents over-alkalization in the presence of millimolar urea and consequent cell death6.

The structure of HpUreI was determined using multicycle anomalous dispersion (MAD; Methods). The structure shows an arrangement of six protomers that form a compact hexameric ring (Fig. 1) about 95 Å in diameter and 45 Å in height. The centre of the hexamer is filled with an ordered lipid plug that forms an asymmetric bilayer with electron density for six lipid tails in the periplasmic leaflet and for 18 tails in the cytoplasmic leaflet. This central lipid plug is reminiscent of those reported for other membrane protein oligomers such as bacteriorhodopsin7. The main inter-protomer contacts are between TMH1 and TMH2 of one protomer and TMH3 and TMH4 of a neighbouring protomer, a region with conserved residues that are probably important for assembly (Fig. 2a). Native gel electrophoresis

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Figure 1 | The HpUreI urea channel hexamer. a, Ribbon diagram of hexamer surrounding the lipids of the central bilayer (purple sticks). The C6 hexamer is generated from the three protomers of one asymmetric unit (green) by the crystallographic two-fold axis (second asymmetric unit in teal). b, Electrostatic potential at the periplasmic hexamer surface computed at pH 5.3, the pH at which the crystals were grown (red, −4kT/e, blue, +4kT/e). The electrostatic potential was calculated with the program APBSmem21. The red arrow pinpoints the entrance to one of six urea channels.
of HpUreI confirms that it is a hexamer (Supplementary Fig. 4), in contrast to the previously postulated trimer10. An electron microscopy 9-Å projection map of a Bacillus cereus amide channel, also a member of the AmiS/UreI family, revealed a similar hexameric arrangement11. Thus, the hexamer probably represents the physiological state rather than a crystallization artefact.

Sequence analysis had predicted six transmembrane segments with periplasmic location of both amino and carboxy termini, generating two periplasmic loops (PLs), one between transmembrane helix (TMH)2 and TMH3 (PL1) and another between TMH4 and TMH5 (PL2), as well as a short periplasmic C-terminal segment12. The crystal structure shows that each HpUreI protomer is a twisted bundle of six slightly tilted transmembrane helices whose inward-facing side chains define a central channel with a unique arrangement of residues (Fig. 2).

Each protomer exhibits non-inverted three-fold pseudosymmetry as demonstrated by the high structural similarity after 120° and 240° rotations around an axis through the centre of the channel (Fig. 3). The repeating motif is a helical hairpin composed of a pair of anti-parallel TMHs connected by a short cytoplasmic loop. However, closer helix–helix packing is observed between helices from adjacent repeated motifs, namely TMH2 and TMH3, TMH4 and TMH5, and TMH1 and TMH6 (Fig. 2a). The mitochondrial ADP/ATP exchanger has a clear internal threefold tandem repeat of a sequence motif with its amino-acid sequence13, whereas the three-fold pseudosymmetry of HpUreI became apparent only after the crystal structure had been determined. Sequence alignments suggest that this motif is present in eubacteria and archaea (Supplementary Fig. 11), implying an ancient evolutionary origin.

The channel is lined by conserved residues that identify the solute pathway in the AmiS/UreI channel superfamily, and specifically the pathway for urea through HpUreI. The beginning of TMH1 is recessed within the helix bundle and starts with Met1, whose ordered side chain was crucial for selenomethionine phasing. TMH1 contains 3 of the 11 channel residues that are conserved in the AmiS/UreI superfamily (Fig. 2 and Supplementary Fig. 11), all facing inwards where they define one side of the channel. The longest helix, TMH2, is near the lipid-filled centre, set back from the channel of the hexamer outside the urea pathway, with one conserved residue. This helix starts more than two helix turns earlier than predicted4. On reaching the periplasmic side, TMH2 continues for several helical turns until the disordered section of PL1, which contains the engineered His6 tag. At this point, TMH2 reaches a height above the bilayer that is similar to the height of PL2. TMH3 is situated inwards of TMH2 and is a major contributor to the urea pathway with four conserved residues in the channel, including Phe 84 and Tyr 88, which form part of two constriction sites in the channel (Figs 2 and 4). TMH3 contacts TMH2 of the same protomer as well as the cytoplasmic half of TMH2 from a neighbouring protomer. TMH4, although part of the repeating structural hairpin motif with TMH3, barely contacts TMH3 and instead is in close contact with TMH5. TMH4, PL2, TMH5, cytoplasmic loop 3 and TMH6 constitute the outer, bilayer-facing edge of the HpUreI hexamer. TMH5 contains five tryptophan residues, three of which (Trp 146, Trp 149 and Trp 153) are highly conserved. These line the urea path, with the aromatic side chains of Trp 149 and Trp 153 being the main components of the two constriction sites (Figs 2b and 4). In the middle of the membrane, the side chain of Glu 177 of TMH6 is in a hydrophobic environment and is thus predicted to have an elevated pKs of 6.7 and to be protonated at pH 5.3, the pH of crystallization. This residue is conserved only in the UreI family of AmiS/UreI members (Supplementary Fig. 12), implying a role in urea transport. It is located to the side of the channel and in a suitable position to hydrogen-bond to urea. Beyond the C-terminal end of TMH6, the last five residues of HpUreI are not helical but bend back towards PL2, where the C-terminal segment is tucked under PL2.

There is probably no high-affinity urea-binding site in the channel, because there is no saturation up to 100 mM urea when expressed in oocytes4, and the half-saturation concentration in proteoliposomes has been reported to be 163 mM (ref. 10). Although neither structurally nor mechanistically related, DvUT from Desulfovibrio vulgaris, a homologue of the mammalian urea transporters, shows half-saturation.

Figure 2 Residues lining the channel. Residues conserved in the AmiS/UreI superfamily (aligned in Supplementary Fig. 11) are clustered in the channel and at the protomer interface. a, View from the periplasm showing the conserved residues of protomer A (gold ribbon) and the open channel of protomers B and C (green and blue ribbons, respectively) when only the protein backbone is shown. For clarity, only selected side chains are labelled. Residues conserved in all members of the AmiS/UreI superfamily are white; additional residues conserved in the subset of known urea channels (including HpUreI) are yellow. The arrow points to the proton-sensing periplasmic loop 2 (PL2) of protomer A. b, View parallel to the membrane, with fully conserved residues (white) clustering in the middle of the channel pore, and residues additionally conserved in the urea channels in yellow. The red arrows show the regions of urea entry (top, periplasmic side) and exit (bottom, cytoplasmic side).
concentrations of 2.3 mM urea. However, it is constructed entirely differently, featuring a long (16-Å), narrow hydrophobic channel with inverted two-fold symmetry also found in the mammalian urea transporter UT-B14. Both of these contain phenylalanine residues at the entry and exit points of the channel.

HpUreI has much larger periplasmic loops than other members of the Ams/UreI superfamily, and proton-gated closure of the channel, affected by mutations of histidine residues or carboxylates in the periplasmic domain, could be due to loop occlusion of the periplasmic vestibule. A similar effect has been shown for the FocA formate channel, but here histidine protonation closes, rather than opens, the channel. For HpUreI at pH 5.3, PL1 is disordered as a result of its engineered His insertion, and PL2 is oriented away from the channel (Fig. 1a), suggesting an open conformation for unimpeded periplasmic entry of urea into the periplasmic vestibule. Urea enters through this irregularly shaped vestibule, which begins roughly at the height of the bilayer edge, defined by a set of largely hydrophobic side chains including Leu 2, Tyr 76, Trp 142 and Trp 146 (Fig. 2b). Next on its way to the cytoplasm, urea must pass through two constrictions, one on each side of Glu 177. Constriction site 1 on the periplasmic side of Glu 177 is defined by the side chains of conserved Leu 6, Phe 84 and Trp 149 (Figs 2b and 4b). Just beyond Glu 177 is constriction site 2, defined by the side chains of conserved Leu 13, Thr 87, Tyr 88, Leu 152 and Trp 153 (Figs 2b and 4b). Trp 153 has its aromatic plane oriented perpendicular to the channel axis, restricting the passage of solutes, and is therefore a candidate for a functional mutation (Supplementary Fig. 8). Its indole side chain can reorient about $\chi_2$ because of minimal steric restraints, enabling the passage of urea and other amides. Similar flexibility of other aromatic side chains in the channel would allow transient $\pi-\pi$ stacking of their side-chain surfaces or NH-...\pi-electrostatic interaction with planar urea to permit selective passage, while at the same time preventing acidification of the cytoplasm by blocking the passage of protons or hydronium. The constrictions in the middle third of the protomer are probably the selectivity filter, which discriminates tenfold between urea and thiourea in oocyte studies. On the cytoplasmic side of constriction site 2, the channel widens again, with Tyr 104 and the hydrogen-bonded pair of Asn 16 and Asn 33 at the beginning of a funnel-shaped vestibule, allowing the exit of urea into the cytoplasm.

Figure 3 | Structural conservation of a two-helical hairpin motif. The internal three-fold axis of pseudosymmetry is oriented perpendicular to the bilayer (black line) and is the result of a threefold tandem non-inverted repeat of a helical hairpin motif (TMH pairs 1 and 2, 3 and 4, and 5 and 6). In this side view, one HpUreI protomer (gold ribbon) has been overlaid on itself by rotations of 120° (green ribbon) and 240° (white ribbon) around the three-fold axis through the centre of the channel pore. The root mean squared deviations for pairwise overlays of the backbone atoms are 1.52 Å for TMH1 and TMH2 onto TMH3 and TMH4; 1.13 Å for TMH3 and TMH4 onto TMH5 and TMH6; 1.76 Å for TMH5 and TMH6 onto TMH1 and TMH2, and 1.98 Å when all are superimposed simultaneously. Structural conservation is stronger for the cytoplasmic half of the channel. Analysed with the program SymD.

Figure 4 | Views of the HpUreI channel traversed by urea. a, Side view with periplasm on top, showing the shape of the channel in yellow and residues conserved in the UreI family (Supplementary Fig. 12). Constriction site 1 is formed largely by the side chains of Leu 6, Phe 84 and Trp 149. On the other side of Glu 177, constriction site 2 is defined by the side chains of Leu 13, Thr 87, Tyr 88, Leu 152 and Trp 153. TMH1 and TMH2 have been removed for clarity. Blue arrows indicate the positions of cross-sections shown in b. b, Close-ups of constriction sites 1 and 2 viewed from the periplasm. c, Channel activity of mutants: mutating Trp 153 to Ala or Phe (constriction site 2) changes selectivity from 4.2:1 (urea/thiourea) for the wild type to 1.5:1 and 1.2:1 for the Ala or Phe mutants, respectively. Mutations at Tyr 88 and Trp 149 inactivate or severely impair the channel, whereas mutation Phe 84→Leu retains discrimination ($n = 10–20$; error bars indicate s.e.m.). d, Western analysis showing nearly equivalent expression for the different mutants.
Previously, mutations of protonatable residues in the periplasmic domain identified residues important for proton sensing or gating\(^{11}\). Here we tested the effect of mutations Phe 84→Leu and Trp 149→Phe in the periplasmic constriction site 1 (Fig. 4b) and Tyr 88→Phe and Trp 153→Ala/Phe in the cytoplasmic constriction site 2 (Fig. 4b) on the discrimination between urea and thiourea (Fig. 4c). A property common to HpuUreI. DvUT and UT-R\(^{13,14,16}\). The rate of uptake of urea or thiourea was measured in Xenopus oocytes. Western analysis confirmed equal expression levels of mutant and wild-type proteins (Fig. 4d). Phe 84→Leu retained the discrimination but with decreased uptake. In the cytoplasmic constriction site, Trp 153→Ala retained urea transport but lost much of the discrimination between urea and thiourea (Fig. 4c). The selectivity for urea over thiourea is therefore largely determined by Trp 153 in the cytoplasmic constriction site 2 of the channel. The buffering capacity generated by proton activation of HpuUreI coupled with urea hydrolysis by the very high levels of urease was estimated on the basis of purified HpuUreI (Supplementary Information)\(^{17}\).

Channel opening also correlates with the acid-dependent recruitment of urease to the cytoplasmic side of the membrane, dependent on HpuUreI and essential for survival at pH 3.0 (ref. 18). About two-thirds of the urease is recruited to the plasma membrane on the basis of post-sectioning immunoelectroscopy at pH 5.0 (ref. 19), confirmed by SDS gel analysis of the membrane fraction of \(\text{H. pylori}\), which also demonstrates activation of the membrane-recruited urease\(^{20}\). Measurement of cytoplasmic pH changes in wild-type and ureI deletion mutants also showed that HpuUreI increases the bilayer permeability of CO\(_2\) and NH\(_3\)/NH\(_4\)\(^+\), providing a physiological role for this association by allowing rapid backflow of these molecules to the periplasm\(^{20}\), thus increasing the rate of periplasmic buffering. These data indicate that a conformational change in the membrane domain of HpuUreI may occur with acidification that is transmitted to its cytoplasmic surface.

In contrast with other neutral-solute membrane channels, such as aquaporins\(^{20}\) and mammalian urea transporters\(^{13,14}\), which are constructed of two homologous antiparallel halves that generate a long, narrow channel, the parallel three-fold pseudo-symmetric architecture of HpuUreI defines a previously unobserved shorter hourglass-shaped channel with a central urea filter and no inverted symmetry. Thus, although both HpuUreI and mammalian urea transporters homologous to DvUT selectively allow the passage of urea, the three-dimensional structures and mechanisms of selectivity are distinct. This first three-dimensional structure of a channel from the AmiS/UreI superfamily reveals a previously unobserved fold with a unique channel architecture able to filter polar organic solutes selectively. As the structure of a validated target for \(\text{H. pylori}\) eradication it may guide the discovery of small-molecule inhibitors, providing the possibility of monotherapy without the use of conventional antibiotics.

**METHODS SUMMARY**

**Crystallography and structure determination.** HpuUreI was expressed in Escherichia coli cells, then purified and crystallized in a mixture of octylglucoside and decylmaltoside, supplemented with E. coli polar lipids (Avanti). The structure was solved using SeMet MAD phasing, followed by threefold non-crystallographic averaging, solvent flattening, phase extension, model building and refinement.

**Functional assays.** Blue native gel electrophoresis indicated a native hexameric structure for HpuUreI. The relative rates of transport of urea and thiourea were measured as uptake of tracer in cRNA-injected Xenopus oocytes with various mutations.

Full Methods and any associated references are available in the online version of the paper.
METHODS

Engineering a His6 tag into HpUreI. DNA encoding HpUreI was isolated by PCR from Helicobacter pylori strain J99. A His6 tag was introduced into the protein at various locations to facilitate purification. The engineered proteins with a His6 tag at the N terminus, in the first periplasmic loop (PL1), the second periplasmic loop (PL2) or at the C terminus were expressed in Xenopus oocytes and tested for channel activity. Wild-type HpUreI showed urea uptake of 29.04 ± 1.35 pmol per oocyte per 10 min (mean ± s.e.m.; n = 9) and the PL1 His6 tag (after Gly61) showed an uptake of 22.36 ± 0.60 pmol per oocyte per 10 min (mean ± s.e.m.; n = 8). Mutants with the His6 insertion at the N terminus, in PL2 or at the C terminus, were inactive. HpUreI with the His6 tag inserted in PL1 (HpUreI6HisPL1) was subsequently used for expression, purification and crystallization.

HpUreI expression and membrane isolation. Small-scale expression. pET101-HpUreI6HisPL1 was transformed into E. coli C43 (Avidis S.A.). For small-scale expression and crude membrane isolation, bacterial cultures were grown to a density of 0.8 and then induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). After 3 h of induction, cells were harvested by centrifugation at 1,500 g for 10 min. The pellet was resuspended in a solution of 50 mM Na2HPO4, pH 7.4, 1 mM EDTA, 30 μg/ml DNaseI and sonicated to lyse the cells. Cell debris was removed by centrifugation (10,000 g, 10 min) and membranes were collected (100,000 g, 45 min) and resuspended in the same buffer without DNaseI (140–50 μl).

Small-scale expression data was collected from a rare larger crystal that took more than 5 months to grow. Three-wavelength MAD diffraction data were collected in 10° wedges at beamline 12-2 at SRL (Supplementary Table 1). SeMet MAD phasing. The program SHELXC25 was used to determine the approximate resolution cutoff for the anomalous signal (when the correlation coefficient between the anomalous differences at the peak and remote wavelengths decreased to below 30%). SeMet sites were initially obtained using the program SHELXD44. Of the nine possible sites (three promoters in the asymmetric unit with three SeMet sites per promoter, including all three N-terminal SeMet residues), eight sites were located. From the symmetry of these eight sites, a ninth site could be located, leading to the localization of all selenium anomalous scatterers. The boundary of the HpUreI hexamer and individual helices were readily apparent in the resulting low-resolution maps; however, unsurprisingly, detail of the maps and phasing power was poor. To improve the phases, the program autoSHARP25 was used. The figure of merit from autoSHARP phasing was used to determine the high-resolution cutoff for the experimental phases. A cutoff of 0.3 for the figure of merit of acenric reflections was used for this estimation, suggesting useful phases to about 4.7 Å resolution (Supplementary Table 2).

Phase improvement and extension. To improve and extend the initial experimental MAD phases, solvent flattening, three-fold non-crystallographic symmetry (NCS) and multi-crystal averaging of data from crystals with different c-axis lengths were performed. To obtain initial matrices for NCS averaging, 18 ideal helices were modelled into the experimental electron density representing the average structure in the asymmetric unit. Subsequently the NCS matrices from molecule A to molecule B and from molecule A to molecule C were obtained by the SSM superpose function of the program COOT26. A mask was placed around the electron density of molecule A and threefold NCS averaging and solvent flattening (68.9% solvent) were performed with the program DM27, while NCS matrices were refined. Phases were extended in an iterative fashion from 4.5 Å to 3.5 Å by using small resolution increments with the programs DM and DMulti27. At this point the right-handed twist of the helices and density for some of the larger side chains could be seen.

Model building and refinement. Density representing each of the six transmembrane helices was cut out with the program PHENIX28. An ideal helix was built into the density, if required, using the program cylinders representing each helix. Helix 6 contains a π-bulge and was built by hand with the program COOT. Cytoplasmic loops 1 and 2 (CL1 and CL2) and periplasmic loop 2 (PL2) were initially modelled into density with the program RAPPER28. In places where the correct sequence could not be built initially, alanine was used temporarily. To generate the other two molecules of the asymmetric unit, the previously identified NCS matrices were used, followed by rigid-body refinement with the program PHENIX. The model was refined iteratively by cycles of manual adjustments with the program COOT and refinement with the program PHENIX, using TLS refine-ment with NCS and secondary structure restraints.

On analysing the data with the University of California Los Angeles anisotropy server29, the data were found to be severely anisotropic with an effective resolution of 3.1 Å in the α* and β* directions, but only 3.5 Å in the c* direction. The data were ellipsoidally truncated and rescaled to minimize the inclusion of poor dif-fraction data. The model was refined with the newly truncated data by using jelly body refinement with the program REFMAC31, leading to significantly improved electron density maps that allowed further model improvement. Because of

X-ray data collection and data reduction. Single crystals were mounted in nylon loops and flash-cooled in liquid nitrogen. X-ray diffraction data collection was conducted at 100 K by collecting 180 diffraction images 1° in width. Data were integrated, scaled and merged with the program XDS20 (Supplementary Table 1). Because of the large variability of the c-axis length (135–152 Å), data could not be merged between multiple crystals, making experimental phasing more challenging. After diffraction data from hundreds of heavy-metal-soaked crystals had been collected without yielding interpretable maps, we resorted to selenomethionine (SeMet) phasing, despite the low abundance of methionine in HpUreI J99 (three residues out of 195, counting the N-terminal methionine). Although fluorescence scans of the initially very small SeMet crystals indicated significant incorporation of selenium, selenium sites could not be located in more than 50 MAD and single-wavelength anomalous dispersion data sets collected with 30° wedges. In successful MAD data sets was collected from a rare larger crystal that took more than 5 months to grow. Three-wavelength MAD diffraction data were collected in 10° wedges at beamline 12-2 at SRL (Supplementary Table 1). SeMet MAD phasing. The program SHELXC25 was used to determine the approximate resolution cutoff for the anomalous signal (when the correlation coefficient between the anomalous differences at the peak and remote wavelengths decreased to below 30%). SeMet sites were initially obtained using the program SHELXD44. Of the nine possible sites (three promoters in the asymmetric unit with three SeMet sites per promoter, including all three N-terminal SeMet residues), eight sites were located. From the symmetry of these eight sites, a ninth site could be located, leading to the localization of all selenium anomalous scatterers. The boundary of the HpUreI hexamer and individual helices were readily apparent in the resulting low-resolution maps; however, unsurprisingly, detail of the maps and phasing power was poor. To improve the phases, the program autoSHARP25 was used. The figure of merit from autoSHARP phasing was used to determine the high-resolution cutoff for the experimental phases. A cutoff of 0.3 for the figure of merit of acenric reflections was used for this estimation, suggesting useful phases to about 4.7 Å resolution (Supplementary Table 2).

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HpUreI crystallization. HpUreI protein at 10 mg/ml in Superose 12 column buffer was dialuted to give a solution comprising 1.57 mg/ml HpUreI protein, 40 mM NaCl, 1 mM TCI, 10 mM CaCl2, 7% PEG 400, 0.05% decylmaltoside, 2.25% octylglucoside, 0.8 mg/ml E. coli polar lipids (Avanti) and 35 mM MES pH 5.3. This mixture (3.5 µl) was used for hanging-drop diffusion over a reservoir (0.5 ml) of 20% PEG 400 in 0.1 M MES pH 5.3. Crystals grew in 3–4 months at 11°C and were dehydrated by raising the PEG 400 concentration in the reservoir in increments of 3% at 2-day intervals until the final PEG 400 concentration in the well solution was 33%. The crystal used for collection of the native data set is shown in Supplementary Fig. 1.
disorder, no model was built for the majority of periplasmic loop 1 (PL1, residues 59–73), which contains the engineered His, insertion.

A final round of refinement was performed with the program REFMAC against the non-truncated data with two TLS groups in each protomer (residues 1–146 and 147–195), tight NCs restraints and a jelly body value of 0.01. The final structure was refined to 3.26 Å with few Ramachandran outliers, small deviations from ideal geometry and predominantly preferred side-chain rotamers (Supplementary Table 1). SeMet anomalous difference maps. To validate the sequence assignment of the HpUreI crystal structure, additional methionine residues were engineered into various sites in the HpUreI sequence and labelled with SeMet. These proteins were crystallized and their anomalous differences Fourier maps were inspected. For all five engineered sites, the location of the anomalous difference peak was less than 1 Å from the site of the methionine sulphur atom of the final model. The sites were Ile 14, Ala 148, Thr 155, Leu 173 and Ile 191, in addition to the endogenous sites Met 1, Met 14 and Met 127, the last of these being situated in PL2.

Estimation of the number of HpUreI molecules per bacterial cell by western blotting. Bacterial proteins were size-fractionated by SDS–tricine polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane, followed by immunodetection by enhanced chemiluminescence (Amer sham). The anti-HpUreI antibody used for detection and quantification was generated in rabbit (against PL1 between TMH2 and TMH3 (CEGAEDI AQVSHHLTSFYGPATG)). Immuno blots were digitized (ScanMaker 1800; Microtek) at a resolution of 600 dpi. The scanned images were analysed with Kodak 1D software and the amount of HpUreI was quantitated with purified HpUreI as the reference. There are 5.3 ng of HpUreI per μg total protein (Supplementary Fig. 2) in 5.4 × 10^10 cells (measured by colony-forming units). The molecular mass of HpUreI is 21.7 kDa. There are therefore 0.24 pmol of HpUreI per 5.4 × 10^10 cells; 0.24 pmol of HpUreI per 6.0 × 10^16 (5.4 × 10^10 cells) is equivalent to about 27 000 channels per cell.

Urease activity assay. Bacteria grown on tryptica soy agar (TSA) plates were suspended in 1 ml of 1 M phosphate buffer to a final D_200 of 0.01. Urease activity was measured radiometrically. Bacteria were added to 100 mM sodium phosphate buffer containing 5 mM KCl, 138 mM NaCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose and 5 mM [14C]urea with a specific radioactivity of 10^4 Ci/m mole (urea). Urease activity was calculated from the rate of urea hydrolysis and the number of channels per cell.

Urease flux estimation. To estimate the urea flux per channel per second, the total urease activity of H. pylori cells was measured under HpUreI-mediated flux conditions. On the assumption that all urea entering the cell is hydrolysed by the large amount of cytoplasmic urease (about 8–10% of total protein) and knowing the number of HpUreI molecules per cell (27 000; see above), the lower bound of the flux was estimated to be about 200 urea molecules per HpUreI channel per second at 5 mM medium urea, calculated from the rate of urea hydrolysis and the number of channels per organism.

This is a tenfold higher influx of urea than that due to unfacilitated diffusion across phospholipid bilayers (4 × 10⁻⁶ s⁻¹ (ref. 33)) and it agrees with the tenfold increase in urease activity observed when HpUreI is open. This calculation does not take into account the roughly fourfold increase in urease activity when incubated at acidic pH (ref. 6); hence the rate could be as low as 1 000 s⁻¹.

NH₃ production due to urea influx through HpUreI. Using the calculated flux rate and the number of channels per cell from above it was estimated that at least 5.4 × 10^10 urea molecules are transported through HpUreI per cell per second at gastric urea concentrations. At pH 5.0, with HpUreI fully open, this can generate about 30 mM NH₃ per second neutralizing capacity in a bacterium with a volume of 0.6 fl.

Blue native gel electrophoresis of HpUreI. E. coli membranes were treated with 2% n-dodecyl-β-D-maltopyranoside (DDM) and the soluble fraction was bound to TALON resin. After washing with buffer containing 20 mM imidazole, HpUreI was eluted with a buffer containing 150 mM imidazole, 50 mM sodium phosphate pH 7.4, 150 mM NaCl and 0.01% DDM. The eluate was concentrated on Amicon filters with 10-kDa cutoff to about 5 mg ml⁻¹. Protein sample (5 μl) was mixed with 2 × sample buffer (100 mM Tricine, 30 mM Bis-Tris pH 7.0, 30% glycerol, 0.02% DDM). The cathode buffer was 50 mM Tricine, 15 mM Bis-Tris pH 7.0, and the anode buffer was 50 mM Bis-Tris pH 7.0. The electrophoretic shift of proteins was achieved by including Coomassie blue G250 dye in the cathode buffer. The running conditions were as follows: initial concentration of Coomassie blue G250 was 0.02% in the cathode buffer and the gel was run for 30 min at 100 V and for a further 2 h at 200 V. The concentration of dye was then decreased to 0.002% and the gel was run for a further 2 h at 300 V. High-molecular mass markers (catalogue number 17-0445-01; Amersham), BSA and trypsin inhibitor (Sigma) were mixed with the same sample buffer (Supplementary Fig. 3).

Solute uptake experiments in Xeposopus laevis oocytes. Genes encoding wild-type HpUreI (H. pylori strain 43504) and His₆-tagged HpUreI (H. pylori strain 399 with the His₆ tag in PL1) were cloned into the pcDNA3.1 (Invitrogen) and PET101 (Invitrogen) vectors, respectively. For the experiments on site-specific mutants, substitutions were introduced into the gene encoding His₆-tagged HpUreI with the use of the QuickChange method (Stratagene). Capped and poly(A)-tailed DNA (cRNA) was prepared with the mMessage mMach 7.1 Ultra Kit (Ambion). cRNA (50 nl, 1 μg μl⁻¹) was injected into each oocyte (Ecoocyte BioScience). Oocytes were incubated for 2 days at 18 °C in Barth’s solution (88 mM NaCl, 2.4 mM NaHCO₃, 1 mM KCl, 0.4 mM CaCl₂, 0.3 mM Ca(NO₃)₂, 0.8 mM MgSO₄, 10 mM HEPES-Tris pH 7.5). Oocytes were transferred to new vials containing 0.5 ml of Ringer’s solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂) buffered by 20 mM MES at pH 5.0 for comparison between urea and thiourea uptake through the open HpUreI channel. The transport reaction was started by the addition of 100 μM [14C]urea or 100 μM [14C]thiourea. After 10 min of incubation at 20 °C the solution was aspirated to terminate transport and the oocytes were washed twice with ten volumes of ice-cold radioisotope-free Barth’s solution. Oocytes were transferred to individual scintillation vials and dissolved in 10% SDS before the addition of scintillation cocktail.

Western analysis of mutant expression levels. Ten oocytes were solubilized by pipetting into buffer containing 0.5 ml of 0.1 M NaCl, 0.02 M Tris–HCl pH 7.6 and 1% Triton X-100. The yolk was spun down by centrifugation at 20,000g for 5 min and the supernatant was aspirated with care, to minimize contamination with floating lipid. Supernatant (20 μl) was size-fractionated by SDS–tricine polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane followed by immunodetection with alkaline phosphatase (Promega). The anti-HpUreI antibody used for detection was generated in rabbit against PL1 between TMH2 and TMH3 (CEGAEDI AQVSHHLTSFYGPATG). All mutants were expressed at similar levels (Fig. 4d).

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