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Helicobacter pylori Urease May Exist in Two Forms: Evidence from the Kinetic Studies

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Purified *Helicobacter pylori* urease displayed a sigmoid curve in the plot of velocity versus [S] at urea concentrations less than 0.1 mM. Under conditions where preservatives, glycerol, or polyethylene glycol (PEG) were added to the enzyme reaction, the substrate hydrolysis was consistent with Michaelis–Menten kinetics, with a K_m of 0.21 ± 0.06 mM and a V_{max} of 1,200 ± 300 μmol min^{-1} mg^{-1}. However, at saturating substrate concentrations, the kinetic parameters of *H. pylori* urease were unaffected by the presence of the preservatives, and enzyme catalysis conformed to Michaelis–Menten kinetics. The Hill coefficients of the enzyme-catalyzed urea hydrolysis in the presence and absence of PEG were 1 and 2, respectively. Based on these findings, we suggest that *H. pylori* urease may exist in aggregated and dissociated forms, each with intact function but differing kinetics that may be of importance in maximizing urea breakdown at varying urea concentrations *in vivo*.

Keywords: *Helicobacter pylori*, urease, kinetics

Helicobacter pylori infection is the main cause of various gastric diseases such as gastritis, gastric lymphoma, peptic ulcers, and stomach cancer [13]. At least half of the world's population is estimated to be infected by this bacterium [11]. The efficacy of pharmacologic therapies directed to eradicate this organism has been limited not only by its resistance apparatus but also by an incomplete understanding of its pathogenic mechanisms [1, 14, 19].

One of the main hallmarks of *H. pylori* is its ability to survive in a hostile acidic environment. The mechanism by which this bacterium resists occasional acid shock is tightly associated with its constitutive urease production [4]. Ubiquitous cellular distribution of vast amounts of urease allows *H. pylori* cells to survive at an acidic pH condition by producing a protective ammonium cloud from urea. This enzyme is also produced by various microorganisms and has been implicated in the pathogenesis of kidney stones, pyelonephritis, hepatic coma, ammonia encephalopathy, and peptic ulceration [12]. We previously reported that an occasional switch between *H. pylori*'s vegetative and coccoid forms is accompanied by a marked change in the distribution of cellular urease. For instance, at the end of the late stationary phase when the switch occurs most actively, the urease pool almost completely disappears from the medium [10]. This suggests that cytoplasmic urease may be the factor enabling *H. pylori* to resist acid shock.

Microbial urea transport systems have extremely low K_m values (10 to 30 μM urea) [12]. The high affinity states adopted by urea transport systems are likely a result of scarce urea availability *in vivo*. Therefore, to assimilate urea efficiently, cytoplasmic urease must also have quite a high affinity for the substrate. In fact, *H. pylori* urease has a considerably lower K_m (0.1 to 0.3 mM) than general microbial ureases, which have K_m values ranging from 10 to 100 mM. *H. pylori* urease is a metalloenzyme composed of two different subunits in equal ratios and displays cooperativity between the subunits [9]. In general, most bacterial ureases are composed of heteropolymeric forms with molecular masses ranging from 200 to 250 kDa [12], and are structurally similar to the jack bean homotrimer [*i.e.*, $(\alpha\beta\gamma)_3$]. By contrast, native *H. pylori* urease aggregates have consistently been found to be significantly larger, with molecular masses of 380 to 600 kDa [5, 6, 9, 17], and have been shown to be related in three-dimensional structure [*i.e.*, $(\alpha\beta)_6$] to the jack bean homohexamers, which have a tendency to associate together into highly ordered disc stacks [2, 5]. Interestingly, hexameric jack bean urease was shown to be reversibly and rapidly halved or dissociated by urease preservatives such as glycol or glycerol without a significant change in activity [3]. Incidentally,

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we observed that the enzyme activity of *H. pylori* urease was appreciably increased by these preservatives, suspecting that this enzyme could actively be dissociated with differential kinetic behavior. To explore the kinetic properties of this enzyme, we purified urease from *H. pylori* and examined the effect of preservatives on enzyme kinetics. *H. pylori* urease has previously been reported to obey simple Michaelis–Menten kinetics [5, 12]. In our study, the enzyme displayed sigmoid kinetics at low urea concentrations while displaying normal hyperbolic kinetics in the presence of preservatives known to induce urea dissociation into functional subunits. This indicates that *H. pylori* urease may actually exist in two forms with different kinetic properties.

H. pylori ATCC 49503 was cultivated in a highly humidified 10% CO_2 incubator for 2 days at 37°C (pH 6.8) on either agar plates or in 1-l Erlenmeyer flasks with brain heart infusion medium containing 5% virus-free horse serum (Difco and GibcoBRL Life Tech., U.S.A., respectively); to avoid bacterial contamination, an antibiotic mixture of 1.5 mg vancomycin, 3.5 mg colistin, and 6 mg nystatin per liter was added to the medium [7].

H. pylori urease was purified as follows: Briefly, freshly harvested bacterial cells were washed once to remove any medium proteins, using a hand homogenizer with 20 mM HEPES-Tris buffer (pH 6.8). After centrifugation at 9,000 ×*g* for 10 min, the precipitated cell pellet was harvested by vortex mixing with deionized distilled water (A_{660}, 0.05) for 30 min before centrifugation. After repeating this water treatment step, more than half of the total cellular urease could be extracted. The water extract was concentrated using ammonium sulfate and then chromatographed on a phenyl-Sepharose column. Active fractions eluting at approximately 0.8 M ammonium sulfate were collected. These fractions were subjected to gel filtration with Sephadex G-200 and then to chromatography with Q-Sepharose at pH 7.4. In the latter procedure, urease was present as a single peak that eluted at 0.1 M NaCl.

Two urease assay methods were used in this study. For continuous assay, portions of the reaction mixture containing a urease sample, 0–5 mM urea, 10 mM phenol red, and 5 mM HEPES-Tris (pH 6.8) were dispensed into cuvettes (1-cm path length). The linear change relative to time at 560 nm was observed at room temperature up to an OD value of 0.5 [15]. If necessary, a modified Berthelot method [18] was also used; portions (~0.1 ml) of the enzyme samples were added to the above HEPES-Tris buffer containing ~5 mM urea to give a final volume of 0.9 ml. After incubation for 10 min at 37°C, aliquots were taken, added to 0.1 ml of phenol-nitroprusside and alkaline hypochlorite (Sigma, St. Louis, U.S.A.), and left at room temperature for 10 min to form indophenol blue. The absorbance was then read at 625 nm.

One unit (U) of enzyme specificity was defined as 1 μmole of urea hydrolyzed per microgram of protein per minute. The protein concentration was determined with a Bradford assay kit (Sigma), using bovine serum albumin as the standard.

Table 1. Effects of various agents on *H. pylori* urease activity.

Additive	Optimum conc. (%)	OD at 625 nm[a]
Glycerol	15	0.42 ± 0.05
PEG	0.5	0.38 ± 0.03
n-Octylglucoside	0.1	0.37 ± 0.08
Triton X-100	0.05	0.36 ± 0.1
SDS	0.02	0.34 ± 0.1
None	-	0.31 ± 0.02

The enzyme was reacted for 10 min at 37°C in the presence and absence of additives, with 0.05 mM urea in 5 mM HEPES-Tris buffer (pH 6.8). [a]Initial velocity (v_o); extrapolated ΔOD values per minute of ammonia/ammonium production from urea. Data represent the means±standard errors from a triplicate assay.

While studying *H. pylori* urease, we found that the enzyme activity appreciably increased under certain concentrations of various agents, including glycerol, polyethylene glycol (PEG), *n*-octylglucoside, Triton X-100, and SDS (Table 1). In particular, glycerol or PEG is a common urease preservative that inhibits enzyme precipitation [3]. However, the enzyme was irreversibly inactivated by acid (pH 4) or 2-mercaptoethylamine, although this effect was less for 2-mercaptoethanol. From these observations, we suspected that the enzyme function would persist even after dissociation into subunits, unless critical amine moieties in the active center of the enzyme are disturbed [8]. Based on these data, we hypothesized that *H. pylori* urease could freely alternate between aggregate and dissociated forms with differential kinetic behavior.

To study the enzyme kinetics, we purified urease from *H. pylori*; from the integrated densities of the SDS–PAGE gel profile, subunits of the purified urease from *H. pylori* were shown to be stoichiometrically equal, with estimated molecular masses of 63,000 ± 2,000 and 29,000 ± 1,000 Da (Fig. 1). From gel filtration on a Sephadex G-200 size exclusion column, the purified *H. pylori* urease was estimated to have a native molecular mass of 580,000 ± 50,000 Da (data not shown). Taken together, the structure of the purified enzyme may be a hexameric aggregate, consistent with the reported stoichiometry of $(29–63\ kDa)_6$ for *H. pylori* urease [5, 9, 12]. We then examined the enzyme kinetics in the presence and absence of the preservative. When the rate of urea hydrolysis was plotted as a function of urea concentration, we found that native urease exhibited a sigmoid curve at low urea concentrations ≤0.1 mM. In the presence of glycerol or PEG, however, the sigmoid pattern changed to a rectangular hyperbola, and urea hydrolysis was consistent with Michaelis–Menten kinetics (Fig. 2). A straight line was therefore given from the Lineweaver–Burk plot, providing a K_m of 0.21 ± 0.06 mM and a V_{max} of 1,200 ± 300 μmol min^{-1} mg^{-1}. It is worth

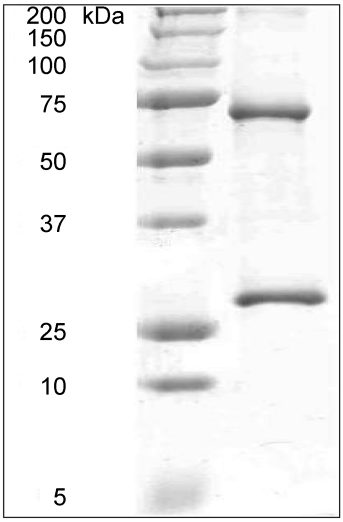

Fig. 1. SDS–PAGE of the purified *H. pylori* urease.
Lanes; left, molecular mass marker proteins; right, an active fraction eluted at 0.1 M NaCl from the Q-Sepharose column chromatography.

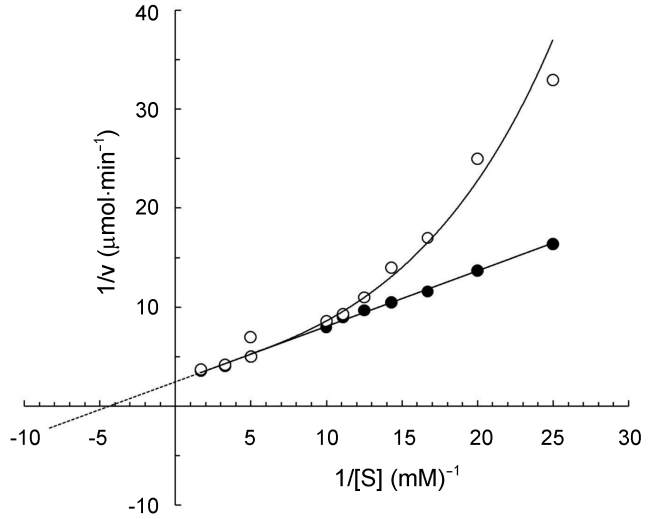

Fig. 3. Lineweaver–Burk plot for urease-catalyzed hydrolysis of urea.
Initial substrate concentrations varied from 0.04 to 0.6 mM. Enzyme concentration, 0.3 μg/ml. Note that the plot of native *H. pylori* urease (○) at [S] ≤0.1 mM presents a characteristic pattern of cooperativity, but then overlapped with the PEG-added plot (●) at high substrate concentrations.

noting that the plot of native enzyme at [S] ≤0.1 mM exhibited a characteristic pattern of cooperativity, but it overlapped with the additive-added plot at high substrate concentrations (Fig. 3). This indicates that, under saturating substrate conditions, the enzyme conforms to Michaelis–Menten kinetics regardless of the presence of preservatives.

To estimate the degree of enzyme cooperativity, we determined Hill coefficient numbers (n_m) in the presence and absence of PEG. For native *H. pylori* urease, the Hill plot was a straight line with a slope of approximately 2, which was 2-fold larger than the slope in the presence of PEG, indicating that native *H. pylori* urease exhibits positive cooperativity between the αβ's hexamer at low substrate concentrations (Fig. 4). On the other hand, *H. pylori* urease in the presence of PEG satisfied the Michaelis–Menten equation with no cooperativity, suggesting that a functional enzyme with hyperbolic kinetics could exist. At certain SDS or Triton X-100 concentrations, however, the enzyme activity rapidly disappeared without a fixed half-

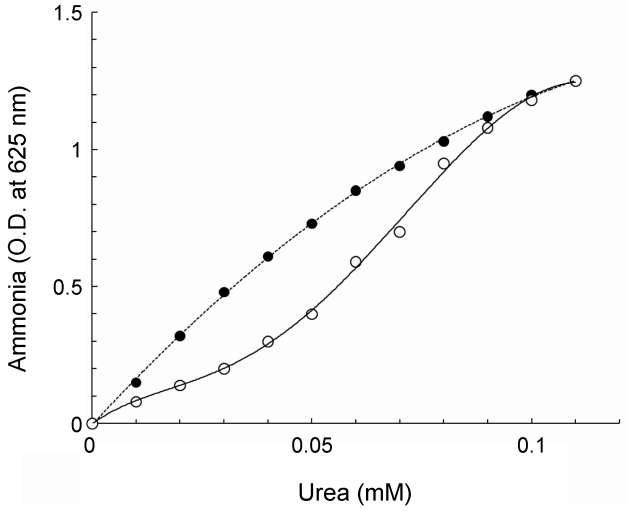

Fig. 2. Effect of preservative on the plot of velocity versus [S] for *H. pylori* urease.
The enzyme was reacted with urea ≤0.1 mM in the presence (●) and absence (○) of 0.5% PEG for 10 min at 37°C, from which initial velocities were extrapolated. The resulting values were plotted against the substrate concentration at which they were determined.

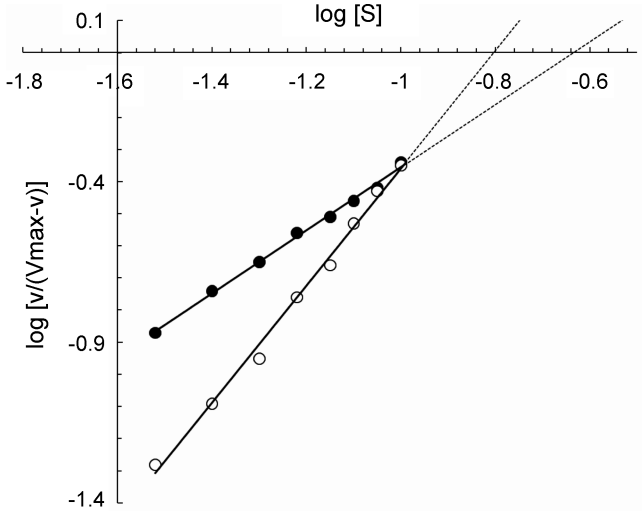

Fig. 4. Determination of enzyme cooperativity by Hill plot.
The rates of urea hydrolysis in the presence (●) or absence (○) of 0.5% PEG by *H. pylori* urease at varying substrate concentrations (0.03 to 0.1 mM) were measured and the data values were used to compute variables of axes in the Hill equation.

life of exponential decay (data not shown). It seems that there would be a concentration limit beyond which the detergents may cause enzyme denaturation, likely *via* an unstable transient state of monomeric heterodimer (*i.e.*, αβ), as suggested by Stark *et al*. [16]. By virtue of the enzyme stability in the presence of glycerol or PEG, we were able to determine the molecular size of the PEG-treated *H. pylori* urease; from size-exclusion chromatography, the main activity was eluted at approximately the half-mass of the native one, with an estimated value of 300,000 ± 30,000 (data not shown). Taken together, *H. pylori* urease with no cooperativity may be multimeric and exist naturally, along with the aggregated form; perhaps dissociation and reassociation occur freely [*i.e.*, between $(\alpha\beta)_3$ and $(\alpha\beta)_6$], similar to the plant urease that readily dissociates into half-units that retain activity [3].

In summary, the purified *H. pylori* urease exhibited sigmoid kinetics with positive cooperativity at urea concentrations ≤0.1 mM, but adopted a hyperbolic curve consistent with Michaelis–Menten kinetics in the presence of preservatives. Based on the kinetics of urease in the presence of the preservatives, the two forms of urease [*i.e.*, $(\alpha\beta)_3$ and $(\alpha\beta)_6$] may well exist in *H. pylori*. The hyperbolic kinetics adopted by the dissociated form of urease may allow for maximal urea assimilation by *H. pylori* under acidic conditions and at low urea concentration.

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References

1. Argent, R. H., R. J. Thomas, D. P. Letley, M. G. Rittig, K. R. Hardie, and J. C. Atherton. 2008. Functional association between the *Helicobacter pylori* virulence factors VacA and CagA. *J. Med. Microbiol.* **57:** 145–150.
2. Austin, J. W., P. Doig, M. Stewart, and T. J. Trust. 1991. Macromolecular structure and aggregation states of *Helicobacter pylori* urease. *J. Bacteriol.* **173:** 5663–5667.
3. Blattler, D. P., C. C. Contaxis, and F. J. Reither. 1967. Dissociation of urease by glycol and glycerol. *Nature* **216:** 274–275.
4. Clyne, M., A. Labigne, and B. Drumm. 1995. *Helicobacter pylori* requires an acidic environment to survive in the presence of urea. *Infect. Immun.* **63:** 1669–1673.
5. Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blazer. 1990. Purification and characterization of urease from *Helicobacter pylori*. *J. Biol. Chem.* **265:** 9464–9469.
6. Evans, D. J., D. J. Jr. Evans, S. S. Kirkpatrick, and D. S. Graham. 1991. Characterization of the *Helicobacter pylori* urease and purification of its subunits. *Microb. Pathog.* **10:** 15–26.
7. Gang, J. G., S. K. Yun, K. M. Choi, W. J. Lim, J. K. Park, and S. Y. Hwang. 2001. Significance of urease distribution across *Helicobacter pylori* membrane. *J. Microbiol. Biotechnol*. **11:** 317–325.
8. Hausinger, R. P. 1993. *Biochemistry of Nickel*, pp. 23–57. Plenium Press, New York.
9. Hu, L. T. and H. L. T. Mobley. 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. *Infect. Immun.* **58:** 992–998.
10. Ki, M. R., S. K. Yun, K. M. Choi, and S. Y. Hwang. 2003. Potential and significance of ammnonium production from *Helicobacter pylori*. *J. Microbiol. Biotechnol*. **13:** 673–679.
11. Langenberg, M. L., G. N. J. Tytgat, M. E. I. Schipper, P. J. G. M. Rietra, and H. C. Zanen. 1984. *Campylobacter*-like organisms in the stomach of patients and healthy volunteers. *Lancet* **i:** 1348.
12. Mobley, H. L. and R. P. Hausinger. 1989. Microbial ureases: Significance, regulation, and molecular characterization. *Microbiol. Rev.* **53:** 85–108.
13. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* **330:** 1267–1271.
14. Rokkas, T., D. Pistiolas, P. Sechopoulos, I. Robotis, and G. Margantinis. 2007. The long-term impact of *Helicobacter pylori* eradication on gastric histology: A systemic review and meta-analysis. *Helicobacter* **12 (Suppl. 2):** 32–38.
15. Ruiz-Herrera, J. and J. Gonzalez. 1969. A continuous method of urease activity. *Anal. Biochem*. **31:** 366–374.
16. Stark, R. M., J. Greenman, and M. R. Millar. 1995. Physiology and biochemistry of *Helicobacter pylori*. *Br. J. Biomed. Sci.* **52:** 282–290.
17. Turbett, G. R., P. B. Hoj, R. Horne, and B. J. Mee. 1992. Purification and characterization of the urease enzymes of *Helicobacter* species from humans and animals. 1992. *Infect. Immun.* **60:** 5259–5266.
18. Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Biochem.* **39:** 971–974.
19. Yamaoka, Y. 2008. Roles of the plasticity regions of *Helicobacter pylori* in gastroduodenal pathogenesis. *J. Med. Microbiol.* **57:** 545–553.