Adherence Protects the Binding Sites of Helicobacter pylori Urease from Acid-Induced Damage

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Abstract: Colonization by Helicobacter pylori partly depends on acid-dependent adherence by urease to gastric mucin. To further verify the relevance of urease adherence to colonization, the influence of acidity on the binding sites of H. pylori urease was investigated. When enzyme-based in vitro ligand capture assays were used, the effect of acidity on the binding site of H. pylori urease was determined against a backdrop medium consisting of acidic buffers simulating the luminal side of gastric mucus. A high degree of stability was exhibited by adherent urease, suggesting a pivotal role by the denatured enzyme in the persistence of the bacterium within the acidified compartment of gastric mucus.

Key words: Helicobacter pylori, Urease, Adherence, Acid

Helicobacter pylori infects about half the world’s population (6) and is the most important etiologic agent of human gastric ulcer and gastric malignancies (1). The remarkable ability of the organism to resist gastric acidity is a multifactorial phenomenon involving both urease-independent (3, 8) and urease-dependent mechanisms. The organism expresses an unusually high level of urease that ultimately occupies a strategic position on the bacterial surface that may be related to the essentiality of this enzyme in colonizing the acidic mucus layer of the gastric mucosa. A neutralophile, H. pylori, can maintain metabolic activity in acidic condition from a minimum of about pH 3.5 (9). With the recent demonstration of bioadherence by urease (4), an open question emerged regarding the significance of this adherence on the binding structures of the native or denatured urease enzyme or to colonization as a whole. Optimum adherence by the denatured enzyme to gastric mucin was shown to be acid-functional with significant binding observable from pH 5.0 down to pH 3.0 (4) that is well within the pH range compatible with viability of the organism. The catalytic activity of unbound extracellular urease persists down to pH 4.0 below, which is known to be irreversibly abolished (2, 10). Nevertheless, the effect of acidity on the adhesive structure of the urease enzyme remains to be verified in relation to any role that this enzyme may play relevant to colonization of an acidic gastric wall. The present study therefore profiled the effect of acidity on the adhesive site and enzymatic activity of urease.

The procedures followed for initial isolation of the H. pylori strain No. 130 from a patient with gastritis, preparation of stock culture, mass bacterial propagation, two-step affinity purification of urease to homogeneity using mild conditions, and its biotinylation were exactly as previously described (4). After a single freeze-thaw cycle, the affinity-purified urease enzyme undergoes spontaneous denaturation (abolation of enzyme activity) and was used in this form for in vitro adherence assays. Mucin from crude mucus taken from a 2-month-old weanling piglet was purified and biotinylated exactly as described previously, using a biotin to protein ratio of 734:1 based on a mucin molecular mass of 2 X 10^6 (4).

The quantitative effect of acidity on the compositional integrity of biotin-labeled urease amines was analyzed by using an enzyme-based microplate assay with a lower detection limit of about 1 ng/ml. Two independent tests with duplicate wells per test sample were conducted. Immulon 2 microtiter wells were coated with biotinylated urease (50 µl/well in concentration that would adsorb about 400 ng/ml), using carbonate-bicarbonate coating buffer, and subsequently blocked with 3% bovine

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharides; PBST, 20 mm phosphate buffered saline + 0.05% Tween-20.
serum albumin. Test wells were then treated at 37°C for 240 min with 50 µl/well of buffers containing 0.1 M phosphate and 0.34 M NaCl preadjusted to different pH levels. To confirm the validity of the assay method in demonstrating amine degradation by acid, control wells were treated with 3% pepsin-fortified buffers in 0.1 M phosphate, 0.34 M NaCl (Japanese Pharmacopoeia standard for artificial gastric juice). At specific time points, wells were washed 5 times with 20 mM phosphate, 0.15 M NaCl, and 0.05% Tween-20 (PBST), pH 7.0, to quench degradation of urease. At the end of the last incubation period, plates were coated with 3% bovine serum albumin and probed with streptavidin-horseradish peroxidase conjugate (Zymed), followed by ELISA as described previously (4). The amount of residual urease in test wells was determined against a standard calibration curve by using samples of known concentrations coprocessed with each test plate.

To determine the effect of acid treatment on the adherence of mucin to urease, the microplate-based format above was modified into an enzyme-linked ligand capture assay having a comparable level of sensitivity. Buffers used for the treatment of urease-coated test wells consisted of 20 mM phosphate, 0.15 M NaCl, and 0.05% Tween-20 (PBST). To confirm the validity of the assay method in demonstrating acid degradation of urease binding sites, buffers used for control wells also contained 3% pepsin. Duplicate tests were conducted with two wells per sample in each test. Briefly, 5 µg/ml native urease was coated onto ELISA plates as above and treated with the above buffers of different pH at 37°C for 4 hr, after which the plates were transferred to 4°C until the 24th hr to avoid drying and nonspecific adherence. To stop urease degradation at specific time points, wells were washed 5 times with a pH 7.0 PBST wash solution. After blocking the plates with 3% bovine serum albumin, biotinylated mucin in suitable concentration that would bind about 400–500 ng/ml in control untreated wells was allowed to bind at pH 4.0 for 1 hr at 37°C. After being washed 5 times with 200 µl/well of pH 4.0 PBST, the plates were processed as in ELISA. Another set of wells with immobilized urease was incubated with mucin without acid pretreatment. For this purpose, urease-coated wells were incubated with labeled mucin using PBST, pH 4.0, as adhesion medium over a similar time period as above. Adherent biotinylated mucin was probed with horseradish peroxidase, and plates were processed as in ELISA.

Based on the time-dependent cleavage rate of urease amines tagged with biotin, the urease protein exhibited a remarkable degree of stability when treated with acidic buffers over a 4 hr period. The time-related curves seen at pH 2.0 to 7.0 (Fig. 1a) indicated that acidity as low as pH 2.0 failed to alter the compositional integrity of the enzyme. On the other hand, the pepsin-fortified control buffers rapidly degraded urease over the same time period (Fig. 1b), indicating the validity of the method for the demonstration of degradation in vitro, but it has little in vivo relevance because the polymeric gastric mucin is relatively impermeable to pepsin.

A significant reduction in adherent mucin was noted when urease was pretreated with pepsin-free buffers of pH 2 to 4 after 24 hr of incubation (Fig. 2a). A more deleterious effect on mucin adherence was attained within 1 hr among pepsin-fortified control wells (Fig. 2b), indicating the validity of the assay for the detection of urease degradation. The reduced binding capacity after 24 hr shown in Fig. 2a possibly resulted from alteration in net electrostatic charges because of the protonation of surface-exposed polypeptides. This finding predicted that the longer the exposure to pH 4.0, urease would bind less mucin. This was not seen, however, in the parallel

![Fig. 1](image-url). Effect of acidity on amino group composition of urease. 1a: A streptavidin-horseradish peroxidase probe failed to detect quantitatively significant cleavage of biotin-coupled urease amines after 4 hr of acid treatment. 1b: Pepsin-fortified control for samples in Fig. 1a showing progressive reduction in biotin-coupled urease amines as a function of time and acidity. Results are presented as percentage of urease amines (ng/ml) detected compared with nontreated control. The following symbols refer to the pH of buffer used to treat urease for 1 hr at 37°C (● = pH 2.0, ○ = pH 3.0, ▲ = pH 4.0, △ = pH 5.0, * = pH 6.0, ■ = pH 7.0).
experiment with urease that had not been pretreated with acid (Fig. 3). By an outright incubation of urease and mucin at pH 4.0 without acid pretreatment, a time-related increase was noted in adherence rate. When the incubation period was extended from 1 hr to 24 hr in the same pH 4.0 medium, a gradual saturation of urease binding sites occurred instead of a reduction in the proportion of bound biotinylated mucin.

The finding on acid stability of urease amino groups shown in Fig. 1 is consistent with a role by denatured urease in mediating H. pylori adherence to mucin in acidic medium. The latter event occurring in vivo is more closely simulated by the conditions of experiments shown in Fig. 3, where urease was immediately exposed to pH 4.0 in the presence of mucin. The data presented in Fig. 3 were surprising and in contrast to those in Fig. 2, where acidity exerted a destructive effect on unbound urease. In the experiments associated with Fig. 3, mucin molecules with partially adherent segments at 1 hr of incubation (detached during washing at this time point) may have been brought gradually into closer and stronger association with the remaining urease binding sites until the 24th hr of incubation. This may explain the trend toward saturation of the urease binding sites over time, as shown in the figure. These findings suggest in unambiguous terms the high-efficiency preservative effect of adherence on urease structural binding sites that were apparently shielded from the disruptive effect of protonation.

The urease enzyme undergoes spontaneous denaturation in vitro (unpublished observations) and most likely much more so in vivo where it may be exposed to gastric acidity of about pH<4.0. Therefore its catalytic activity after adherence cannot be expected to persist for an indeterminate period. In fact, the existence of a urease-independent mechanism of acid resistance (3, 8) suggests that H. pylori may need to survive at times without the benefit of the pH regulating ammonia derived from extracellular urealysis. After abrogation of enzymatic activity by acid, the urease protein continues to express its built-in function as a mucin-targeted bacterial ligand (Fig. 3). The presence of inactivated urease protein on the surface of H. pylori appears to have a natural purpose beyond mere molecular ornamentation of the outer membrane. Based on the finding that urease binds mucin and LPS (5), the hexameric urease with

Fig. 2. Effect of preexposure of native urease to pH buffers on urease binding sites. 2a: When biotinylated mucin was allowed to bind at pH 4.0 for 1 hr onto wells coated with native urease that were preexposed to acidic buffers, adherence was reduced as a function of preexposure time and degree of acidity. 2b: Parallel tests using pepsin-fortified medium as control showed a more rapid and more drastic reduction in urease-mucin adherence than in Fig. 2a. Results are presented as a percentage of urease amines (ng/ml) detected compared with nontreated control. The following symbols refer to the pH of buffer used to treat urease before the incubation of mucin at pH 4.0 for 1 hr at 37 C (*=pH 2.0, ○=pH 3.0, △=pH 4.0, △=pH 5.0, *=pH 6.0, /=pH 7.0).

Fig. 3. Adherence of mucin to native urease not preexposed to pH buffers. When urease was not pretreated with acid (●) and allowed to bind mucin at pH 4.0, the binding sites were saturated until the 24th hr of incubation (155%) with quantitative adherence arbitrarily set at 100% at 1 hr of incubation. There was rapid degradation of control urease (○) incubated in pepsin-fortified buffer.

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multivalent binding sites may therefore reversibly cross-link bacteria to mucin and promote its retention therein against peristaltic movements of the gut during intervals of acidity around pH 3.0 to 4.0. The persistence of urease-mucin interaction despite prolonged acidity (Fig. 3) suggests a major role by the adhesive component of urease in the persistence of \textit{H. pylori} in its natural niche. This is consistent with our earlier targeting experiments in mice where acid-dependent urease-homing polysaccharides significantly suppressed colonization by \textit{H. pylori} (5). Similarly, this is consistent with the capacity of \textit{H. pylori} to survive and persist in acidic medium as low as pH 3.0 by maintaining a constant transmembrane proton motive force (7).

In conclusion, the adherence by \textit{H. pylori} urease protein under acidic condition protects its own binding structures that may partially account for the long-term persistence by the bacterium in the stomach mucosa despite acidity. When the present findings and the recognition of intracellular (10) and extracellular catalytic activity of urease as crucial elements in the viability of \textit{H. pylori} in acidic medium are considered, it is worth examining the essential role of this primordial bacterial enzyme in steady-state host colonization from the point of view of its hetero-functionality, that is, as an enzyme cum adhesive activity.

References


