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Purification and Characterization of the Urease Enzymes of *Helicobacter* Species from Humans and Animals

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The urease enzymes of *Helicobacter pylori*, *H. mustelae*, *H. felis*, and *H. nemestrinae* have been purified to homogeneity by affinity chromatography and characterized. The native urease enzymes of the four organisms were found to be almost identical, with a pI of 6.1 and molecular masses of 480 to 500 kDa, as determined by electrophoretic mobility in nondenaturing polyacrylamide gels. Transmission electron microscopy of the native urease showed it to be a molecule approximately 13 nm in diameter, with hexagonal symmetry. Denaturation studies indicated that each urease enzyme molecule was composed of two nonidentical subunits with molecular masses of approximately 64 and 30 kDa. The subunits were present in a 1:1 ratio, suggesting a hexameric stoichiometry for the native molecule. The predicted molecular mass of *H. pylori* urease, based on subunit molecular weight and stoichiometry, is 568 kDa. N-terminal amino acid sequencing of the enzyme subunits from the four species revealed high levels of homology. The large subunits (UreB) were found to be 92 to 100% homologous, and the small subunits (UreA) were 75 to 95% homologous over the first 12 to 20 residues. The high degree of homology suggests a common ancestral origin and an important role for the urease enzymes of these organisms.

Helicobacter pylori is now well established as an important human pathogen, able to cause gastritis, gastric and duodenal ulceration, and possibly gastric carcinoma in humans (39, 41). A related organism, *H. mustelae*, has been associated with gastritis in ferrets (16), and similar findings have been reported for mice experimentally infected with *H. felis* (30). Numerous factors have been proposed to be important in the pathogenesis of diseases caused by these organisms (for a review, see reference 32). Urease production has been shown to be an important colonization factor in a gnotobiotic piglet model (11), and various authors have proposed mechanisms by which urease may play a role in the disease process (23, 33, 48–50). Dunn et al. (10) have characterized the urease enzyme of *H. mustelae* and found it to be similar to that of *H. pylori*, but little is known about the urease enzymes of any other *Helicobacter* sp.

To understand the role that the enzyme plays in the pathogenesis of *Helicobacter* infections, adequate characterization and comparison of their urease enzymes are essential. To our knowledge, this article is the first report of the purification of the urease enzymes of *H. felis* and *H. nemestrinae* and is the first report of detailed characteristics of the enzymes from four *Helicobacter* species.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study were obtained from the following sources: the *H. pylori* isolates NCTC 11637 and 72R (human), RPD2 (rhesus monkey), CP1 (cynomolgus monkey), NAS3 (macaque monkey), BM1 (baboon), and PM1 (pig) were obtained from C. Stuart Goodwin and the Department of Microbiology at Royal Perth Hospital, Perth, Western Australia, and have been described elsewhere (18, 55). *H. nemestrinae* ATCC 49396 was isolated by Bronsdon et al. (3). *H. mustelae* ATCC 43772 was originally isolated by Fox et al. (15). *H. felis* CS1 (ATCC 49179) was originally isolated by Lee et al. (31). All *Helicobacter* isolates were grown on horse blood agar plates for 48 to 72 h at 37°C in a microaerobic environment as described by Goodwin et al. (18) and characterized by colonial morphology, Gram stain appearance, rapid urease hydrolysis, catalase production, oxidase production, nitrate reduction, and pattern of resistance and sensitivity to nalidixic acid and cephalothin in a disk diffusion assay (3, 42).

Preparation of samples and enzyme purification. Cell extracts of bacteria were prepared for electrophoresis by harvesting bacterial growth from 20 blood agar plates into 10 ml of ice-cold 20 mM sodium phosphate buffer (pH 6.8). The suspensions were sonicated for 3 min in 30-s pulses with ice cooling, centrifuged at 5,000 × *g* for 10 min to remove cell debris, aliquoted, and frozen at −70°C. Partially purified urease was obtained after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by eluting the portion of the gel containing the active urease into 10 mM Tris-HCl (pH 8.0) as described previously (14, 55). For affinity chromatography, the cell extracts were filtered through a 0.22-mm filter to prevent clogging of the column.

Samples of *Helicobacter* urease were prepared for use in nondenaturing isoelectric focusing gels by the freeze-thaw method outlined by Tompkins et al. (54). Briefly, 3-day cultures from blood agar plates were harvested into double-distilled deionized water and then lysed by four cycles of freezing (in liquid nitrogen) and thawing (in a 40°C water bath). The cell debris was removed by centrifugation at 5,000 × *g* for 5 min, and the supernatants were used immediately.

MAb production. Monoclonal antibodies (MAbs) were produced as described previously (55). Two fusions were performed. The antigen for the first fusion was partially purified urease from *H. pylori* 72R. An MAb obtained from this fusion was used in an affinity chromatography column to purify the urease of *H. pylori* NCTC 11637, which was used as the antigen for the second fusion. From this, an MAb

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specific for the large subunit was obtained and used in a second affinity chromatography column to purify the urease enzymes of *H. mustelae*, *H. felis*, and *H. nemestrinae*. The specificities of the MAbs are described elsewhere (54a).

Purification of urease by affinity chromatography. Urease was purified by affinity chromatography with MAbs directed against the native urease enzyme (MAb N-4) or, in later studies, against the large urease subunit (MAb SU-1). The MAbs (both immunoglobulin G1) were purified from ascites fluid on a protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column by eluting with 0.1 M glycine-HCl (pH 3) and then neutralizing the eluate with 1% (wt/vol) Tris. The purity of the MAb in the eluate was confirmed by SDS-PAGE. Eluates containing the MAb were pooled, placed in dialysis tubing, concentrated with polyethylene glycol 6000, and then dialyzed overnight at 4°C in coupling buffer (0.1 M $NaHCO_3$ [pH 8.3]). The dialyzed MAbs were mixed with cyanogen bromide-activated Sepharose 4B beads (Pharmacia) and blocked with 1 M ethanolamine (pH 8), and 5 ml of this mixture was packed into a column. Cell extracts of bacteria were passed through the column five times. Urease activity was checked before and after passage through the column by using Christensen's urea agar slopes. After extensive washing with 0.1 M Tris-HCl (pH 8), the enzyme was eluted with 0.1 M glycine-HCl (pH 3). The eluates were neutralized, and purity was examined by SDS-PAGE. Eluates containing two protein bands of 64 and 30 kDa were stored in aliquots at −20°C. Urease enzymes purified by this method will be referred to as affinity chromatography-purified urease (ACP-urease).

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (29) and was used to determine the molecular masses of urease enzyme subunits and for Western immunoblotting. Protein standards (Bio-Rad) were included in each determination of the molecular masses. The standards for SDS-PAGE were myosin (200 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). The buffers and techniques used for nondenaturing PAGE were the same as those for SDS-PAGE except that SDS was omitted from all buffers and solutions, SDS and 2-mercaptoethanol were omitted from the sample buffer, and the samples were not heated prior to electrophoresis. Nondenaturing, linear-gradient PAGE gels were used to estimate the molecular masses of the native urease enzymes. Protein standards (Pharmacia) were included in each determination of molecular mass made with nondenaturing PAGE gels. The standards for these analyses were hog thyroglobulin (669 kDa), horse spleen ferritin (440 kDa), bovine liver catalase (232 kDa), bovine heart lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa). The molecular masses of unknown proteins were determined by constructing standard curves for each gel, plotting the log molecular mass of the protein (vertical axis) against the distance migrated from the interface of the stacking and resolving gels (horizontal axis). The molecular masses of unknowns were calculated by interpolation from the standard curve. For estimates of the molecular mass of the native urease enzyme, a correlation coefficient of −0.995 or greater was required.

PAGIF. Polyacrylamide gel-isoelectric focusing (PAGIF) was performed as described by Robertson et al. (45), with the Bio-Rad vertical polyacrylamide minigel system. Both nondenaturing PAGIF and denaturing PAGIF (containing 8 M urea) were performed. Gels of 0.75 mm thickness were cast for use. The carrier ampholytes used were the Bio-Rad Bio-Lyte 3/10 (for PAGIF over the pH range of 3.7 to 9.2). The acrylamide-bisacrylamide mixture was prepared to give a final concentration of 5% T (total acrylamide concentration) and 3% C (percent cross-linking bisacrylamide of total acrylamide). The isoelectric point (pI) of unknown proteins was determined by interpolation from standard curves (pI of protein against distance migrated) constructed by using proteins of known pI (Bio-Rad). The standard pI proteins were cytochrome *c* (red), 9.6; lentil lectin (three bands), 8.2, 8.0, and 7.8; human hemoglobin c (red), 7.5; human hemoglobin a (red), 7.1; equine myoglobin (brown), 7.0; human carbonic anhydrase, 6.5; bovine carbonic anhydrase, 6.0; β-lactoglobulin B, 5.1; and phycocyanin (blue), 4.65.

Detection of enzymatic activity within gels. Urease activity was detected in PAGE gels either by the urease-specific silver stain (7) or by a pH-dependent staining method (14), except that cresol red (0.1%, wt/vol) was used in place of phenol red. Activity within PAGIF gels was detected as described by Tompkins et al. (54). With the cresol red activity stain, bands of urease activity were detected by flooding the gel with 2% (wt/vol) urea. The positions of the bands were noted, and the gels were then stained with Coomassie blue. By comparing the sites of urease activity from the jack bean urease standard with the sites of jack bean urease protein and other standard proteins visualized with Coomassie blue, the molecular masses of the native urease enzymes of all *Helicobacter* species tested were estimated.

TEM. ACP-urease samples were prepared for transmission electron microscopy (TEM) by adsorption onto Formvar-coated copper grids for 30 s by flotation, blotted dry, washed in water, and negatively stained for a further 30 s with 2% (wt/vol) phosphotungstic acid or 2% (wt/vol) silicon tungstate. Grids were examined with a Phillips 410 transmission electron microscope.

N-terminal amino acid sequencing. ACP-urease was prepared for sequencing by extensive dialysis in double-distilled deionized water (three lots of 6 liters over 72 h) to remove all traces of glycine and then recovered by lyophilization. The samples were resuspended in double-distilled deionized water for sequencing. The N-terminal sequencing protocol is outlined briefly as follows. Automated amino acid sequence analysis was performed in an Applied Biosystems model 470A gas-liquid phase sequencer with trifluoroacetic acid conversion chemistry (24). Phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography on a Zorbax C8 reversed-phase column (Du Pont, Wilmington, Del.) eluted with a discontinuous acetonitrile gradient (57).

AAS. Graphite furnace atomic absorption spectroscopy (AAS) was performed on ACP-urease samples with a Varian (Varian Techtron) AA-175 series atomic absorption spectrophotometer linked with a Varian CRA-90 carbon rod atomizer. Sample volumes of 10 μl were injected into the graphite furnace, dried at 100°C for 60 s, ashed at 800°C for 25 s, and then atomized at 2,200°C for 2 s, with a ramp rate of 400°C/s. Nickel was detected at 232 nm, with a slit width of 1 nm. Standard curves were constructed from solutions of known nickel concentration (0.05 to 0.25 μg/ml), prepared from $NiCl_3 \cdot 6H_2O$ in 0.1 M glycine-HCl (pH 3), which had been neutralized to pH 7 with 1% Tris.

Computer-aided video densitometry. Densitometry was performed with the Bio-Rad model 620 CCD video densitometer, fitted with a 600-nm interference filter (Bio-Rad) and linked to an Apple Macintosh IIx computer running the

Bio-Rad 1-D analyst/Macintosh data analysis software (version 1.00). ACP-urease samples or cell extracts were electrophoresed on Protean II linear gradient (5 to 20%) SDS-PAGE gels or 12% T SDS-PAGE Protean minigels. Gels were stained with Coomassie blue (37) and placed on the glass platen of the densitometer, and single lanes were scanned up to three times. The scan results were averaged, and the peaks were identified manually. A linear baseline value for each scan was determined and subtracted, but the data were not filtered, smoothed, or enhanced prior to peak integration.

RESULTS

Molecular mass of the partially purified urease enzyme and its subunits. Partially purified urease preparations exhibited activity in the elution buffer and on nondenaturing PAGE gels. The urease-specific silver stain was used to identify bands of urease activity in both SDS-PAGE and nondenaturing PAGE gels of cell extracts (55). The molecular mass of the partially purified urease of *H. pylori* was estimated from gels to be 484 ± 12 kDa. The same molecular mass was obtained for urease from cell extracts electrophoresed on nondenaturing PAGE gels silver stained for urease activity and subsequently stained with Coomassie blue to allow visualization of the protein standards. Proteins of the same molecular mass as the partially purified urease were observed in cell extracts of *H. felis*, *H. mustelae*, and *H. nemestrinae* isolates. Isolates of *H. pylori* from four species of monkey and a pig were also found to have a urease with the same molecular mass as was observed for human isolates. With the cresol red stain, the molecular masses of the native urease enzymes of all *Helicobacter* species tested were estimated to be 495 ± 20 kDa. When partially purified urease was heated for 3 min in a 100°C water bath prior to electrophoresis, it appeared as two nonidentical subunits, neither of which possessed urease activity (Fig. 1A).

Molecular masses of the ACP-urease enzyme subunits. With the two affinity columns, the urease enzymes of *H. pylori*, *H. mustelae*, *H. felis*, and *H. nemestrinae* were purified. The ureases of isolates of *H. pylori* from a pig and a baboon were also purified for characterization. None of the ACP-ureases were found to possess enzyme activity. When the ACP-urease samples were examined by reducing SDS-PAGE, two subunits were observed for all four *Helicobacter* species (Fig. 1B). The molecular masses are listed in Table 1. These two subunits were seen to correspond to major protein bands in cell extracts on SDS-PAGE gels. These bands disappeared or were greatly reduced in cell extracts that had been passed through the affinity chromatography columns (Fig. 1A). Following convention (28), we will refer to the large subunit as UreB and the small subunit as UreA. ACP-urease from *H. pylori* isolates recovered from a pig, rhesus monkey, and baboon exhibited subunit sizes of the same molecular masses as those for the urease from *H. pylori* NCTC 11637, isolated from a human.

Subunit stoichiometry by video densitometry. Manual identification of scanned peaks allowed determination of the percentage that the peak associated with each subunit contributed to the total identified area. Scanning of *H. pylori* NCTC 11637 ACP-urease gave an average ratio for the UreB and UreA subunits of 64 and 36%, respectively, while the ratio for *H. mustelae* was 65 and 35%, respectively. When adjusted for the molecular masses of the two subunits (Table 1), the urease subunits give a 1:1 molar ratio. The urease subunits of both *H. felis* and *H. nemestrinae* appeared in the ratio of 57 to 43%. This translates into a molar ratio of 1:1.6. However, the quality of the protein from these two organisms was poorer, and the subunits appeared as diffuse bands which were not as readily quantitated on the densitometer.

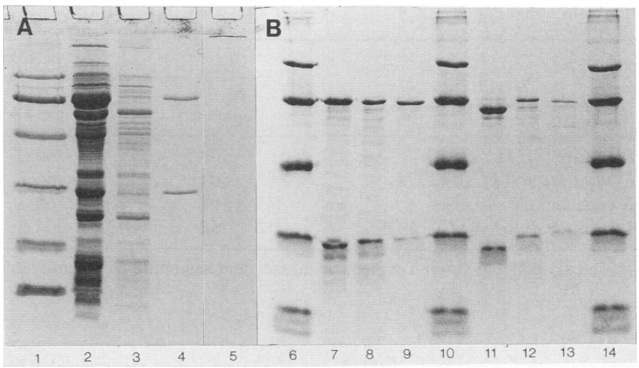

FIG. 1. (A) SDS–12% PAGE gel of *H. pylori* NCTC 11637 stained with Coomassie blue. Lane 1, molecular mass markers; lane 2, crude cell extract; lane 3, cell extract after passage through an affinity column (note the absence of two major proteins at 64 and 30 kDa); lane 4, acid glycine eluate (ACP-urease) from the affinity column (note that a similar appearance is produced when partially purified urease enzyme preparations are analyzed by SDS-PAGE after being heated at 100°C for 3 min); lane 5, unheated partially purified native urease protein preparation. (B) SDS–12% PAGE gel of ACP-ureases from *Helicobacter* species. Lanes 6, 10, and 14, molecular mass markers; lane 7, *H. pylori* NCTC 11637; lane 8, *H. pylori* RPD2; lane 9, *H. pylori* CP1; lane 11, *H. mustelae* ATCC 43772; lane 12, *H. pylori* PM1; lane 13, *H. felis* ATCC 49179. The molecular mass markers are 97.4, 66.2, 42.7, 31, and 21.5 kDa in both panels. A 14.4-kDa marker is also visible in panel A.

pI of the native urease enzyme and the enzyme subunits. Nondenaturing PAGIF was performed to determine the pI of the native urease enzymes, while denaturing PAGIF was used to measure the pI of the individual subunits. When cell extracts of *H. pylori* NCTC 11637 were electrophoresed on nondenaturing isoelectric focusing gels and the urease was detected by staining for urease activity, it was observed that active urease possessed a pI of 6.1 (data not shown). ACP-urease samples were also examined. The ACP-urease enzymes of the four *Helicobacter* species were observed to have identical pI values of 6.3. A pI of 6.3 was also observed for ACP-ureases from *H. pylori* isolates from the rhesus monkey, baboon, and pig (data not shown). Under denaturing PAGIF conditions, two major protein bands were resolved for the ACP-ureases from each of the four *Helicobacter* species. The pI values observed for the UreB subunits ranged from 4.9 to 5.1, while the UreA subunits were found to have a pI from 7.0 to 7.8. The pI values obtained are listed in Table 1.

N-terminal sequence analysis. N-terminal sequencing was performed on the ACP-urease samples without separating the enzyme into its individual subunits. Thus, both subunits were sequenced simultaneously, a method that requires foreknowledge of the sequence of at least one of the two proteins. By comparison with two published *H. pylori* sequences (5, 28), the N-terminal sequences of the ureases of the four *Helicobacter* species were determined. The results of the sequencing are provided in Tables 2 and 3. The N-terminal sequences of both subunits of three *H. pylori* isolates (NCTC 11637, BM1 and PM1) were the same, and all three were in complete agreement with the sequences published by Dunn et al. (9) and Labigne et al. (28). Differences

TABLE 1. Mass and pI of native urease enzymes and subunits

Strain	Mass (kDa)			pI		
	Native enzyme[a]	UreB subunit	UreA subunit	Native enzyme	UreB subunit	UreA subunit
H. pylori NCTC 11637	568	64.3	30.4	6.3	5.1	7.0
H. mustelae ATCC 43772	564	64.8	29.2	6.3	5.0	7.7
H. felis ATCC 49179	597	69.3	30.2	6.3	4.9	7.8
H. nemestrinae ATCC 49396	566	64.3	30.1	6.3	5.1	7.0

[a] Calculated from subunit molecular masses and assuming hexameric stoichiometry.

between the ureases of *Helicobacter* species that could be determined with absolute certainty were the presence of Met-11 instead of Leu in the *H. mustelae* UreA subunit and the absence of lysine at the second residue in both subunits. In the *H. nemestrinae* UreA subunit, A has replaced H at residue 14 and the UreA subunit is unlikely to possess a G at position 17. Although the identity of residue 10 in the *H. felis* UreA urease subunit could not be determined, it was possible to ascertain that it was not Lys.

TEM. The ACP-urease samples of *H. pylori* NCTC 11637, *H. felis*, and *H. mustelae* were visualized by TEM. The urease of *H. nemestrinae* was not examined. Figure 2 shows the ureases from *H. pylori* and *H. felis*. The TEM pictures show the urease enzyme as a round or hexagonal molecule 13.5 nm in diameter, with a darker-staining core which gives the molecule a distinctive doughnut-like appearance. It appears that only a little of the material present in the sample is native urease, and the bulk of the material appeared as large amorphous masses. However, SDS-PAGE and video densitometry indicate that the ACP-urease preparations were pure. The amorphous masses seen are believed to be processing artifacts or aggregations of urease protein, making visualization of discrete urease molecules difficult.

AAS of urease. The urease preparation of *H. pylori* NCTC 11637 used for AAS contained 1.2 mg of protein per ml. From the known protein concentration of the sample used, the number of urease molecules in the 10 μl injected into the furnace could be quantitated. One molar urease was assumed to be equal to 484,000 g/liter; therefore, a sample containing 1.2 mg of urease per ml has a urease concentration of 2.48 μM. From the standard calibration curve for nickel, approximately 82.5 ng of nickel was detected per ml of ACP-urease sample, equivalent to 1.42 μM. Thus, for every 2.48 urease molecules, 1.42 nickel atoms were detected, giving an average of 0.6 nickel atom per urease molecule. If the molecular mass of the native urease enzyme is higher than 484 kDa, about 550 kDa, as has been suggested by Hu and Mobley (26), then the ratio of nickel to urease was 0.5:1.

DISCUSSION

Molecular masses were estimated after SDS-PAGE by silver staining and subsequent Coomassie blue staining of gels. There were no detectable differences between the molecular masses of partially purified native urease from *H. pylori* 72R and the native enzyme in cell extracts of other *H. pylori* isolates, *H. nemestrinae*, or *H. mustelae*, suggesting that all have ureases with a molecular mass of 484 ± 12 kDa. With the cresol red activity stain, the *H. pylori*, *H. mustelae*, and *H. felis* ureases were all observed to have a molecular mass of 495 ± 20 kDa. The PAGE-based values derived from the two staining methods agree closely. The reported molecular masses for the native urease of *H. pylori* vary widely, from 300 kDa (14) to 625 kDa (25). Molecular mass estimations that have been performed with Superose columns gave a molecular mass range of 380 kDa (9) to 625 kDa (34). Estimates by PAGE (with SDS or nondenaturing) have ranged from 300 kDa (14) to 600 kDa (13). The wide variation in the results highlights the inherent difficulties in estimating molecular mass and suggests that there are factors capable of interfering with estimations of the molecular mass of these large proteins, such as the concentration of salts and the presence of other proteins and nucleic acids. Several recent reports have indicated that the molecular mass of *H. pylori* urease is greater than 500 kDa (10, 13, 21, 22, 26, 34). The lower molecular mass observed for the urease in this study may be a result of abnormalities in the protein configuration during electrophoresis or of an effect of the intrinsic charge of the protein, factors known to affect estimates of protein molecular mass during PAGE (6).

Ureases are typically large enzymes composed of one or more subunits and either are multimers (two to six copies) of a single repeating subunit or are composed of three different subunits in a more complex arrangement (35). There have been few reports of urease enzymes composed of two subunits. Early reports (12, 46) that the urease of *Ureaplasma urealyticum* has two subunits have been contested (2, 52). Thus, it would appear that members of the genus

TABLE 2. N-terminal sequences of the large (UreB) subunit of *Helicobacter* ureases

Organism	Sequence[a] (residues 1–20)
H. pylori[b]	M K K I S R K E Y V S M Y G P T T G D K
H. pylori NCTC 11637	M K K I S R K E Y V S M – – – – – – – – – –
H. mustelae ATCC 43772	X^1X^2 K I S R K E Y V S M Y G P T T G(D)K
H. felis ATCC 49179	M K K I S R K E Y V S M – – – – – – – – – –
H. nemestrinae ATCC 49396	M K K I S R K E Y V S M Y G P T T G(D)K

[a] Residues not definitely identified are shown in parentheses. Residues not identified are shown as –. X^1 is likely to be methionine, and X^2 is not lysine (see text for details).

[b] From Dunn et al. (9).

TABLE 3. N-terminal sequences of the small (UreA) subunit of *Helicobacter* ureases

Organism	Sequence[a] (residues 1–20)
H. pylori[b]	M K L T P K E L D K L M L H Y A G E L A
H. pylori NCTC 11637	M K L T P K E L D K L M – – – – – – – –
H. mustelae ATCC 43772	X^1 X^2 L T P K E L D K M M L H Y A G (E) L A
H. felis ATCC 49179	M K L T P K E L D X^2 L M – – – – – – – –
H. nemestrinae ATCC 49396	M K L T P K E L D K L M L A Y A X^3 (D) L A

[a] See Table 2, footnote *a*. X^1 is likely to be methionine, X^2 is not lysine, and X^3 is not glycine (see text for details).
[b] From Dunn et al. (9).

Helicobacter have been confirmed as the first organisms to possess a urease enzyme composed of only two structural subunits. In our previous publication (55), the subunit molecular masses were given as 68.2 and 31.3 kDa. Subsequent determinations have caused us to revise our estimates, and we now believe that the UreB subunit is 64.3 kDa and the UreA subunit is 30.4 kDa.

Densitometric studies reveal that the two urease subunits of *H. pylori* have a 1:1 molar ratio when adjusted for the molecular masses of the two subunits. This indicates that the native urease enzyme must therefore possess a $(UreB:UreA)_n$ structure. An identical molar ratio was obtained for the *H. mustelae* urease subunits and is presumed for the *H. felis* and *H. nemestrinae* ureases. With molecular mass values of 64 and 30 kDa for the urease subunits, a pentamer would have a calculated molecular mass of 470 kDa. The hexamer would possess a molecular mass of 564 kDa. The molecular mass of the native urease enzyme, based on subunit stoichiometry and molecular mass, appears to be 564 kDa, and this suggests that PAGE-derived estimates for the native enzyme have been affected by chemical and/or physical factors. The molecular masses of the native urease enzymes of four *Helicobacter* species, based on the molecular weights of their subunits and on the predicted stoichiometry of a hexamer, are presented in Table 1.

The TEM observations for the dimensions of the urease enzymes correlate with those of Hawtin et al. (22), who reported 10-nm doughnut-like structures for *H. pylori* urease. Similar values have been reported previously by Jones et al. (27) and Newell (38). Blakeley and Zerner (1) have also reported aggregation with jack bean urease molecules as a result of processing of samples for TEM. In their review, the jack bean urease molecule has been observed to be 115 ± 10 Å (11 ± 1 nm) in diameter, with a structure comprising six globular subunits arranged in an octahedral symmetry, giving rise to a staggered stack of two trimers to form a trigonal antiprism. It appears that the native urease enzymes of the helicobacters may possess a similar shape. Figure 2B shows the urease of *H. felis* at the highest magnification obtained. In several of these molecules, it is possible to see a distinct Y-shaped marking, suggesting a trilobar shape. Each of the three lobes may correspond to the UreB-UreA structure, which is equivalent to a single jack bean urease subunit. If this is the case, then the other three lobes, each a dimer, are hidden below the top three lobes. This result also suggests that the *H. pylori* urease enzyme is a hexamer. Not all molecules appear to exhibit this Y-shaped marking. This may be explained by the fact that the urease molecule is likely a nonspherical object, with each molecule presenting a different face for visualization. This phenomenon is dis-

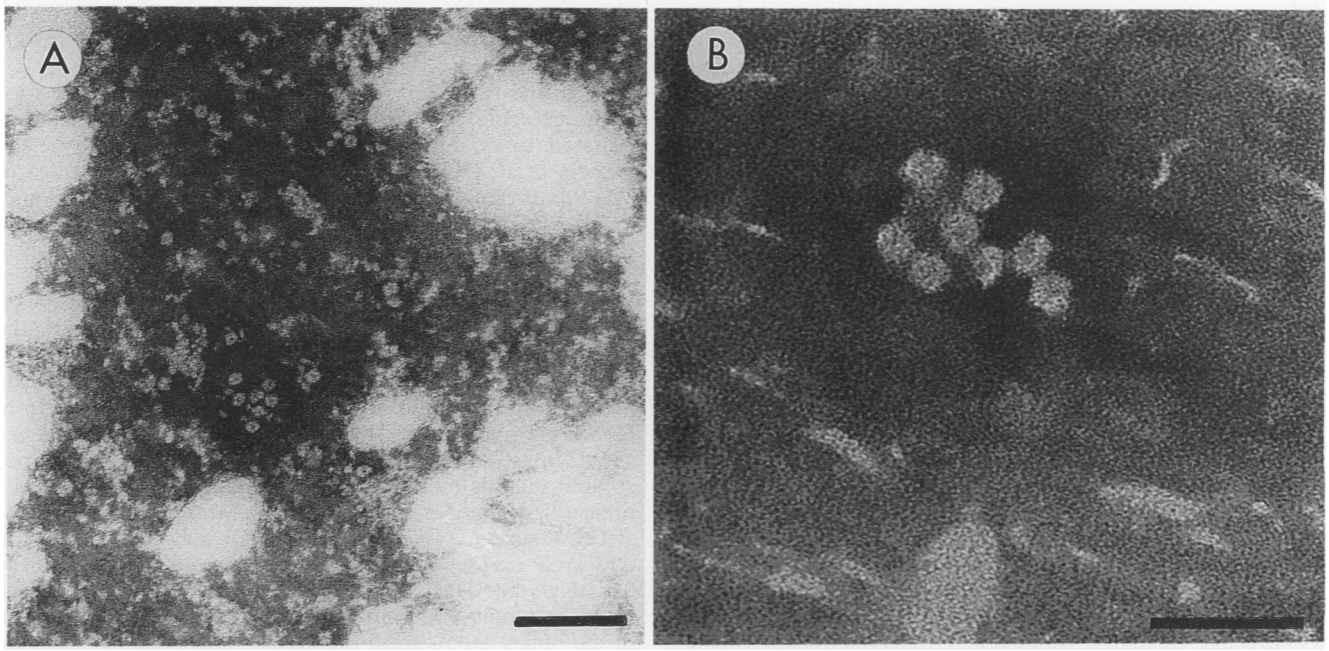

FIG. 2. TEM of ACP-urease. (A) Urease from *H. pylori* NCTC 11637. Magnification, approximately ×147,535. Bar, 100 nm. (B) Urease from *H. felis* ATCC 49179. Magnification, approximately ×398,254. Bar, 50 nm.

cussed in a review by Oliver (40). The intensity of the stain at the site may also play a significant role in the resolution of fine detail.

The pI of 6.1 noted for active urease is close to the values reported elsewhere. Tompkins et al. (54), using agarose isoelectric focusing, reported urease pI values of 5.9 for *H. pylori* for a human and a pig isolate, 5.7 for a baboon *H. pylori* isolate, and 5.4 for *H. mustelae*. Dunn et al. (9) reported the native enzyme to have a pI of 5.99 ± 0.03. More recently, Evans et al. (13) found that the enzyme from *H. pylori* has a pI of 5.93. No differences in the pI values of ureases from the different species were observed in our study, in contrast to Tompkins et al. (54). At the time of that publication, the taxonomic identification of the animal isolates was uncertain, and they had not yet been classified as *H. pylori*.

The pI for ACP-urease was 6.3, 0.2 pI unit greater than that obtained for the native urease. Lack of the nickel ion core may produce the higher pI values for ACP-urease. Effects of this type have been noted by other researchers. As reviewed by Righetti and Drysdale (44), the presence of metal ions or the redox state of the metal ion(s) may alter the surface charge of the molecule, resulting in the presence of anomalous forms of the protein or multiple banding patterns. This phenomenon has been observed in rabbit transferrin (56); the iron-free transferrin molecule had a pI of 6.0, a one iron mole-transferrin complex had a pI of 5.5, and a two iron mole-transferrin complex had a pI of 5.1.

The pI values obtained for the subunits were found to vary according to the species from which the urease was isolated. Variation of only 0.2 pI unit was observed for the UreB subunits, and variation of up to 0.8 pI unit was observed for the UreA subunits, consistent with the greater sequence diversity found (Table 2). The urease subunits of *H. pylori* and *H. nemestrinae* could not be distinguished by the isoelectric focusing data. Isoelectric focusing is a powerful analytical technique that is capable of differentiating proteins that differ by as little as only a few amino acids (43). From the N-terminal sequencing data, it has been shown that differences do occur between the urease subunits and may account for the differences measured.

Four sequences for the *H. pylori* urease subunits have been published. Clayton et al. (5) and Labigne et al. (28) have determined the complete nucleotide and the derived amino acid sequences for both subunits. Dunn et al. (9) have determined the sequence of 20 residues at the N terminus of each subunit, while Hu and Mobley (26) have published the first 15 residues, both based on N-terminal sequencing of the purified proteins. However, when the 20 N-terminal residues of the UreB subunits are compared, inconsistencies were reported at three positions: 6 (R or A), 10 (A or V), and 19 (A or D). No inconsistency in the UreA subunit sequence has been reported. Our data for *H. pylori* are in agreement with the sequences reported by Dunn et al. (9) and Labigne et al. (28).

We could not identify the first residues of either subunit of *H. mustelae* urease because of excessive background from serine, glycine, alanine, methionine, and lysine. The first residues are likely to be methionine, as serine, glycine, and alanine are frequent contaminants in sequencing reactions. The residue at position 2 for both subunits is not lysine. There was one change in the remaining 18 residues analyzed, which occurred at Leu-11 of the UreA subunit, where Met was present. Recently, Dunn et al. (10) have published some of the characteristics of the *H. mustelae* urease enzyme, and we agree with their published N-terminal sequence. They reported that the *H. mustelae* UreB subunit had Ile (rather than the Lys in *H. pylori*) and the UreA subunit had Arg at position 2, but our analysis of these positions was unclear. They reported high homology for the urease subunits of *H. pylori* and *H. mustelae*, with 16 of 17 (94%) residues identical between the UreB subunits and 18 of 20 (90%) residues identical between the UreA subunits.

The UreB subunit of *H. nemestrinae* urease was identical to that described for *H. pylori* urease, while in the UreA subunit, the His at residue 14 has been replaced by Ala. At position 17, only threonine was detected, which is presumed to have originated in the UreB subunit. The residue at that point in the UreA subunit could not be identified, but it was unlikely to be glycine. In *H. felis* urease, the UreB subunit sequence was identical to that of *H. pylori* urease, while for the UreA subunit, one change at amino acid 10 (lysine in *H. pylori*) appeared, but its identity could not be determined.

When the N-terminal sequences of the ureases obtained in this study are compared, it can be seen that there is an extremely high level of homology among them. It may be seen that there was 95 to 100% homology among all of the UreB subunits, while the similarity among the UreA subunits was slightly lower, ranging from 92% (between *H. pylori* and *H. felis* and between *H. felis* and *H. nemestrinae*) to 75% (between *H. mustelae* and *H. felis*). If the overall degree of similarity is compared, it may be seen that *H. pylori* and *H. felis* had the most similar ureases. The least similar ureases were those of *H. mustelae* and *H. felis*. *H. mustelae* and *H. nemestrinae* were the second most distant ureases. When the 16S rRNA sequence homology data provided by Paster et al. (42) are examined, it may be seen that the data obtained here support their conclusions. In comparing this sequence in *H. pylori*, *H. felis*, and *H. mustelae*, they found that *H. pylori* and *H. felis* were the most similar (95.9% homology). Also, the 16S rRNA sequences of *H. pylori* and *H. mustelae* were the most distant (93.7% homology), whereas the ureases of *H. mustelae* and *H. felis* were found to be the most distant in this work, although this finding is based on limited sequence data. They also noted that *H. mustelae* was slightly more closely related to *H. felis* than to *H. pylori*, differing from the comparisons here.

From the AAS data, 0.6 atom of nickel was detected for each molecule of ACP-urease. One other group has attempted to quantitate the nickel content of the urease. Hawtin et al. (20) were able to demonstrate an average of 5.21 atoms of nickel per urease molecule after purification of the enzyme by size exclusion on a Superose 6 fast protein liquid chromatography column. They have proposed, based on results published by Hu and Mobley (26), that the most probable number of nickel atoms per molecule is 6 (1 nickel atom per subunit). These data are in agreement with the results obtained for other urease enzymes, which have been shown to possess either 1 or 2 nickel atoms per subunit. One nickel atom per subunit was reported for the ureases from *Bacillus pasteurii* (4) and *Brevibacterium ammoniagenes* (36). Urease enzymes found to have 2 nickel atoms per subunit include jack bean urease (8), *Klebsiella aerogenes* urease (53), and *Selenomonas ruminantium* urease (19).

The results obtained from the AAS may help to explain why ACP-urease was devoid of activity. It is likely that the 0.1 M glycine-HCl (pH 3) buffer used to elute the urease from the affinity column resulted in permanent dissociation of the nickel atoms from the urease molecule. Taylor et al. (51) reported that the urease activity in *H. pylori* is irreversibly lost at pH 4.5 and below. Furthermore, Schneider and

Kaltwasser (47) have reported that the nickel of *Arthrobacter oxydans* urease was irreversibly released under acidic conditions (pH 5 and below), with a concomitant loss of enzyme activity. Hu et al. (25) have cloned and expressed the urease gene of *H. pylori* in *E. coli*. They showed that the urease subunits were assembled into a molecule with the correct molecular mass and yet did not possess any enzyme activity and postulated that the molecule may be lacking the nickel ions required for activity. Thus, the molecule that they have expressed in *E. coli* appears to be similar to the ACP-urease of this study in that it is fully assembled but nonfunctional.

Physical characterizations of the urease enzymes of *H. pylori*, *H. mustelae*, *H. felis*, and *H. nemestrinae* have shown that the enzymes are similar. All had the same molecular mass and pI for the native urease and were composed of two subunits, a situation that appears to be unique among the ureases reported to date (35). TEM has shown that the molecule has some structural similarities to the hexameric jack bean urease molecule. The two subunits were found to have the same approximate molecular mass and pI in each of the four species. The subunits were purified and found to exist in a 1:1 molar ratio. From TEM observations and the sizes of the subunits and the native enzymes, the native enzymes were likely to be arranged as a hexamer. N-terminal amino acid sequence comparisons have indicated that there is a considerable degree of homology among the *Helicobacter* urease enzymes. These results show that the urease enzyme has been highly conserved among gastric spiral bacteria and suggest that the enzyme has a significant role in the biology of these organisms.

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