The leucyl aminopeptidase from *Helicobacter pylori* is an allosteric enzyme

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This study describes the cloning, genetic analysis and biochemical characterization of a leucyl aminopeptidase (LAP) from *Helicobacter pylori*. A gene encoding LAP was cloned from *H. pylori* and the expressed 55 kDa protein displayed homology to aminopeptidases from Gram-negative bacteria, plants and mammals. This LAP demonstrated amidolytic activity against L-leucine-p-nitroanilide. Optimal activity was observed at pH 8.0 and 45 °C, with *V*~max~ of 232 μmol min~−1~ (mg protein)~−1~ and *S*~0.5~ of 0.65 mM. The data suggest that LAP could be allosteric (*n*_H~2~ = 2.27), with regulatory homohexamers, and its activity was inhibited by ion chelators and enhanced by divalent manganese, cobalt and nickel cations. Bestatin inhibited both LAP activity (IC~50~ = 4.9 ± 9 nM) and *H. pylori* growth in vitro. The results point to the potential use of LAP as a drug target to develop novel anti-*H. pylori* agents.

INTRODUCTION

*Helicobacter pylori* is a microaerophilic, Gram-negative, spiral, flagellated bacterium which causes superficial gastritis, chronic atrophic gastritis, peptic ulcer and gastric cancer (Hopkins et al., 1996; Kreiss et al., 1995). It is prevalent in almost half the world’s population (Cover & Blaser, 1996). No vaccine is available at present and antimicrobial therapy for the infection is a complex issue. Although current optimal first-line treatment, consisting of proton-pump inhibitors and/or bismuth, metronidazole, clarithromycin or amoxicillin (Malfertheiner et al., 2002), is associated with high cure rates, the rising incidence of resistance to the antibiotics increasingly threatens to compromise the efficacy of these eradication regimens (Björkholm et al., 2001). Therapeutic agents directed against *H. pylori* infection will continue to evolve and there is a pressing need for the identification of novel drug targets, such as enzymes vital to the survival of this bacterium. This opportunity exists now as a result of successful sequencing of the genomes of two *H. pylori* strains (Tomb et al., 1997; Alm et al., 1999).

Aminopeptidases, which catalyse the removal of N-terminal amino acid residues from peptides and proteins (Taylor, 1993), play an important role in several physiological processes. It is noteworthy that some of them take part in the catabolism of exogenously supplied proteins (Smid et al., 1991; Booth et al., 1990), and are necessary for the final steps of protein turnover (Lazdunski, 1989; Goldberg & Dice, 1974; Goldberg & John, 1976) and maturation (Lazdunski, 1989; Miller 1975). Bacterial aminopeptidases can be classified based on their catalytic mechanisms: metallo-, cysteine and serine aminopeptidases. The metalloaminopeptidase is predominant in bacteria; its activity is regulated by the presence of divalent metallic cations and may be inhibited by chelating agents (e.g. EDTA) (Thierry & Janine, 1996).

Most of the above studies demonstrate that bacterial aminopeptidases generally show Michaelis–Menten kinetics though they possess a multimeric structure. Very few of them display allosteric kinetics.

Because of their critical role in the life cycle of microorganisms, aminopeptidases are emerging as novel and promising drug targets, especially in the development of new anti-parasitic agents (Niven, 1991; Nankya-Kitaka et al., 1998). Some aminopeptidase inhibitors show good efficacy against parasites such as *Plasmodium falciparum* and *Trypanosoma brucei* (Knowles, 1993; Howarth & Lloyd, 2000). Two other aminopeptidase inhibitors, Bestatin and 1,10-phenanthroline, exhibit notable inhibitory effects on the growth of *Fusobacterium nucleatum* (Rogers et al., 1998). However, aminopeptidase from *H. pylori* has not yet been reported.

In this paper, we describe the cloning, genetic analysis and biochemical characterization of a leucyl aminopeptidase.
(LAP) from a *H. pylori* standard strain (ATCC 43504). The data presented considerably expand our knowledge, which thus far is rather limited, of the peptidolytic capacity of *H. pylori*.

**METHODS**

**Bacteria and culture conditions.** A standard strain of *H. pylori* (ATCC 43504) was kept at −80°C in 10% sucrose and 50% heat-inactivated fetal bovine serum (FBS). It was cultured on Columbia agar (Difco) containing 10% defibrinated sheep blood and then passaged to *Brucella* broth supplemented with 5% FBS, as described by Müller et al. (1991). Plates and tubes inoculated with *H. pylori* were incubated at 37°C under microaerophilic conditions (10% CO2, 5% O2 and 85% N2) for 1–4 days. *Escherichia coli* BL21 (DE3) pLysE was grown at 37°C in Luria broth or agar supplemented with antibiotics when needed.

**Cloning, sequencing and analysis of the LAP ORF.** Using the published sequence of the *H. pylori* genome (GenBank accession number NC_000915; gene HP0570), forward (5’-GAC CAT ATG TTA AAA ATC AAA TTA GAA AAA ACC-3’) and reverse primers (5’-CCC CTC GAG AGC CTT TTTCAA AAG CTC TT-3’) were synthesized that were complementary to the complete coding sequences flanking a *H. pylori* ORF designated ‘probable cytosol aminopeptidase’. Built-in Ndel and Xhol sites are shown by bold type and the initiation codon is underlined. The stop codon was built into the plasmid vector. PCR amplification of genomic DNA from *H. pylori* was carried out in the following order: initial denaturation (95°C, 2 min), 30 cycles of denaturation (95°C, 1 min), annealing (50°C, 1 min), primer extension (72°C, 2 min) and final extension (72°C, 10 min). PCR products were cloned into pET26b (Novagen). The plasmid was sequenced using T7 forward primer and M13 reverse primer, and the result indicated that the ATG start codon was in-frame.

**Hyperexpression of recombinant LAP.** The LAP gene was cloned into pET26B and the construct was transformed into *E. coli* pLysE. The N-terminal polyhistidine-tagged fusion protein was expressed by induction of exponential-phase culture (500 ml; OD600~0.6, as determined by a UV2001 spectrophotometer, Shimadzu) with 1 mM IPTG for 4 h at 37°C with vigorous (300 r.p.m.) shaking. Bacteria were harvested by centrifugation (7000 g, 10 min, 4°C), resuspended in 20 mM Tris/HCl, 500 mM NaCl and 1 mM PMSF, pH 7.9 (20 ml, 4°C), and sonicated with a Branson Sonifer 250 sonicator (Branson Ultrasonics Corp.). Centrifuged (15000 g, 15 min, 4°C) extracts were heated in 65°C water for 10 min, centrifuged again (15000 g, 15 min, 4°C), and peptidases purified on nickel-agarose resin (Novagen) according to the manufacturer’s instructions. Sample purity was evaluated on a Coomassie blue-stained 12% Tris/Tricine SDS-PAGE gel. A Sephadex G200 column (Amersham Biosciences) was used to determine the molecular mass of the enzyme in its oligomerization state. The total column volume was approximately 60 ml, and the standard buffer was 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, with or without 1 mM DTT, at pH 8.0. The metal content of LAP protein was analysed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) at the Contemporary Analytical Center, Nanjing University.

**Enzymic analysis of recombinant LAP.** Kinetic analysis of LAP was carried out according to the procedure of Tan & Konings (1990), with some modifications. The standard reaction mixture contained 50 mM Tris/HCl (pH 8.0), 0.5 μM MnCl2, 15 μg LAP protein and an appropriate amount of L-leucine-p-nitroanilide (l-Leu-p-NA). After incubation at 37°C for 10 min, the enzyme and l-Leu-p-NA were sequentially added to start the reaction. Absorbance at 405 nm was continuously measured for a minimum of 2 min by UV2001 spectrophotometer (Shimadzu). The initial velocity was calculated from the slope of the linear range of an absorbance versus time curve. Vmax was obtained from the substrate-saturation curve. The Hill plot method (Dixon & Webb, 1979) was used to analyse the data to achieve half-saturation constant (S0.5) and Hill number (nH). S0.5 values are defined as the concentration of the substrate that gives 50% maximal activity.

The LAP pH profile was determined by incubating the enzyme (15 μg ml−1, 37°C, 5 min) in constant ionic strength acetic acid/MES/Tris (AMT) buffer [50 mM acetic acid, 50 mM MES and 100 mM Tris/HCl (Ellis & Morrison, 1982), pH 4–11] and 0.5 mM MnCl2 before addition of L-Leu-p-NA (final concentration 5 mM).

The metal ion dependence of LAP was investigated by assaying LAP activity after preincubation of the enzyme (15 μg ml−1, 37°C, 10 min) in 50 mM Tris/HCl, pH 8, containing a given metal chloride (0.01–10 μM) before adding 5 mM substrate.

Temperature dependence of the enzyme was determined by incubating LAP (15 μg ml−1, 15–85°C, 3 min) in 50 mM Tris/HCl and 0.5 mM MnCl2, pH 8, before addition of 5 mM L-Leu-p-NA.

Inhibition of LAP activity by peptidase inhibitors was determined by preincubation of the enzyme (15 μg ml−1, 37°C) with EDTA, PMSF and Bestatin (Sigma) in 50 mM Tris/HCl and 0.5 mM MnCl2, pH 8, for 10 min before addition of the substrate. In the presence of metal ion chelator, MnCl2 was omitted in the assay buffer. The IC50 (the concentration of inhibitor that gave 50% maximum inhibition) of the non-tight-binding reversible competitive inhibitor, Bestatin, was estimated from the inhibition curve.

**Inhibition of *H. pylori* growth by Bestatin.** *H. pylori* (106 cells) was grown in *Brucella* broth medium containing 5% FBS with or without different concentrations of Bestatin under microaerophilic conditions at 37°C for 24 h. The growth of *H. pylori* was determined by measuring OD600 of the growing bacterial suspension using the UV2001 spectrophotometer.

**RESULTS**

**Cloning, sequencing and analysis of the *H. pylori* LAP gene**

The LAP gene isolated from *H. pylori* (ATCC 43504) consists of an ORF of 1491 base pairs that encodes a polypeptide of 496 amino acids with predicted molecular mass of 54 398 Da. According to the nucleotide sequence retrieved from the GenBank database, this gene encodes a polypeptide chain containing an M17 LAP family signature sequence (Rawlings & Barrett, 1995). Sequence alignment analysis with other members of the M17 family suggests the existence of multiple conserved amino acid residues essential to the predicted catalytic activity (Fig. 1a). Phylogenetic comparison of the full-length *H. pylori* LAP sequence with six other M17 LAPS (including homologues from prokaryotic and eukaryotic organisms) indicates that the former is the least evolutionarily divergent member of this family (Fig. 1b). In addition, this enzyme exhibits high identity to other M17 LAP family members: the homology between *E. coli* and *Haemophilus influenzae* is 56% and between *E. coli* and humans 30%, while *H. pylori* LAP only...
possesses 26, 28 and 24 % identity to its counterparts from E. coli, Hae. influenzae and humans, respectively.

Enzymic properties of recombinant H. pylori LAP

The full-length LAP gene was expressed in E. coli as catalytically active, polyhistidine-tagged recombinant enzyme with a yield of 3–6 mg (l bacterial culture)\(^{-1}\). The observed molecular mass of the recombinant LAP (~55 kDa) is consistent with that calculated from the sequence of the affinity-tagged translational product (55 220-96 Da).

The LAP activity eluted in a single, well resolved peak from a Sephadex G-200 column at a molecular mass corresponding to 340 kDa. No activity was eluted at an elution volume corresponding to 55 kDa (the molecular mass of the translation products of H. pylori lap genes). These data may indicate that the translation product associates into catalytically competent homohexamers and suggests that LAP monomers may not be catalytically active. A similar observation has been reported for Leishmania Laps (Morty & Morehead, 2002).

Kinetic studies against L-Leu-p-NA suggest that H. pylori LAP is an allosteric enzyme because the V versus S plots of both uninhibited and Bestatin-inhibited LAP were not hyperbolic but sigmoid (Fig. 2a) (Dixon & Webb, 1979) with an estimated \(V_{\text{max}}\) of 232 \(\mu\)mol min\(^{-1}\) (mg protein)\(^{-1}\). The extinction coefficient for the product p-nitroanilide is 10 \(\mu\)mol (\(A_{405}\))\(^{-1}\). Hill plot analysis (Fig. 2b) gave an \(n_H\) value of 2-268 and an \(S_{0.5}\) of 0-65 mM.

Amidolytic activity against L-Leu-p-NA was optimal at pH 8-0 and still detectable at pH values up to 10 (Fig. 3a). It rapidly declined under moderate acidic conditions (pH 5-0). Activity could be influenced by temperature: it was optimal at 45 °C (Fig. 3b), retaining approximately 60% activity at 100 °C. The enzyme did not precipitate under any of the experimental conditions used in this study.

The LAP exhibited enhanced activity in the presence of...
several metal ions with a rank order of manganese > cobalt > nickel (Table 1). Calcium and zinc showed inhibitory effects at 0-1 mM and abolished enzymic activity at 10 mM. After incubation with the metal-ion chelator EDTA, metal-depleted LAP (apo-LAP) displayed a negligible activity (<3%), whereas PMSF did not demonstrate any effects.

Bestatin is a potent LAP inhibitor, which blocks enzymic action through a slow binding inhibition mechanism (Morty & Morehead, 2002). The IC₅₀ of Bestatin on H. pylori LAP was 49.9 nM (Fig. 4), comparable to that estimated from the substrate-saturation curve (Fig. 2a).

**Inhibition of H. pylori growth by Bestatin**

In the presence of Bestatin, the growth of H. pylori was markedly suppressed and this effect was concentration dependent (Fig. 5).

**DISCUSSION**

We found previously that one anti- H. pylori agent, NE-2001 (Cheng et al., 2003), was able to upregulate the expression of the LAP gene in H. pylori (data not shown). This led us to speculate that LAP may play an important role in the life cycle of this bacterium. To answer this question, we performed a series of experiments relative to the cloning, genetic analysis and biochemical characterization of LAP from a standard strain of *H. pylori* (ATCC 43504) as presented in this paper. To our knowledge, this is the first peptidase identified in *H. pylori*.

The protein encoded by the *H. pylori* LAP gene only possesses 20–30% identity with other members of the M17 family of metallopeptidases. It belongs to an evolutionarily distant group of the M17 LAPs, clustering in its own branch of the phylogenetic tree (together with the plant *Lycopersicon esculentum*) that diverges from *E. coli* and *Hae. influenzae*.
The biological significance of this genetic trait remains to be elucidated.

Optimal amidolytic activity of *H. pylori* LAP was observed against L-Leu-p-NA and, like other M17 LAPs, it exhibited a broad substrate specificity including Met, Arg, Ala, Ile, Val, Phe, Gly and Tyr (data not shown). This is consistent with the relatively simple genome and proteome of *H. pylori*. Since LAP is the only aminopeptidase in this bacterium, its broad substrate specificity ensures adequate release of various amino acids from polypeptides to maintain the life cycle.

Intriguingly, the data suggest that the kinetics of *H. pylori* LAP could be allosteric, which is different from that of most bacterial aminopeptidases, which show Michaelis saturation kinetics (Thierry & Janine, 1996). The only aminopeptidase that has been shown to observe allosteric kinetics is aminopeptidase A from *Lactococcus lactis* ssp. *lactis* (Niven, 1991). Like this metalloaminopeptidase, *H. pylori* LAP, with an $n_H$ of 2.27, is a typical allosteric enzyme in that it relies on positive co-operativity to enhance binding of the substrate. This feature implies that, due to the higher efficiency of allosteric enzymes, *H. pylori* LAP may play a more important role in the life cycle than LAPs in other organisms.

### Table 1. Effects of divalent cations and various reagents on *H. pylori* LAP activity

Data shown are mean ± SD ($n=3$).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
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<td>Control</td>
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<td>EDTA</td>
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<td></td>
<td>10</td>
<td>566 ± 15</td>
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<td>10</td>
<td>380 ± 10</td>
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<td>Ca(II)</td>
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<tr>
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Metalloaminopeptidases exhibit a broad range of metal-ion dependence. M17 LAPs mainly utilize Zn(II) (Carpenter & Vahl, 1973), whereas other aminopeptidases are dependent upon Mn(II) (Cottrell et al., 2000), Fe(II) (D’Souza & Holz, 1999) and Zn(II) (Walker & Bradshaw, 1998). We have demonstrated in this study that *H. pylori* LAP was inactivated by incubation with metal-ion chelators and that its activity was enhanced by Mn(II), Mg(II), Co(II) and Ni(II) at millimolar concentrations. Analysis of the...

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**Fig. 4.** Inhibition of *H. pylori* LAP activity by Bestatin. LAP (15 μg ml$^{-1}$) was preincubated with different concentrations of Bestatin in 50 mM Tris/HCl (37 °C, pH 8.0) prior to determination of enzymic activities. Data shown are means ± SD ($n=3$).

**Fig. 5.** Inhibitory effects of Bestatin on the growth of *H. pylori*. The bacterium ($10^6$ cells) was grown in *Brucella* broth containing 5% FBS, with or without different concentrations of Bestatin, under microaerophilic conditions at 37 °C for 24 h. Growth was measured by OD$_{600}$ using the UV2001 spectrophotometer. Data shown are means ± SD ($n=3$).
metal content of H. pylori by ICP-AES suggests that Zn is most likely the metal cofactor for this enzyme, consistent with the observations on the bovine lens LAP (Carpenter & Vahl, 1973). Two zinc-binding sites of LAP from bovine lens (Kim & Lipscomb, 1993) and from tomato (Gu & Walling, 2002) have been identified. Site 1 readily exchanges Zn(II) for other divalent metal cations including Mn(II), Mg(II) and Co(II). Site 2 binds to Zn(II) more strongly and retains it under conditions that allow exchange of Zn(II) from site 1. It is therefore possible that activation of H. pylori LAP with Mn(II), Mg(II), Co(II) and Ni(II) might result from substitution of the site 1 Zn(II) with these ions. Substitution of the site 1 Zn(II) with Mn(II), Mg(II) and Co(II) has been shown to activate porcine kidney LAP via elevating the $k_{cat}$ (Van Wart & Lin, 1981). Unlike the metal chelators, PMSF did not suppress the activity of this enzyme, suggesting that no serine is involved in catalysis.

Bestatin, a potent competitive inhibitor of aminopeptidase (Wilkes & Prescott, 1985; Taylor et al., 1993), inhibited not only H. pylori LAP activity but also the growth of the bacterium. Because LAP is the only aminopeptidase in H. pylori, the bacterial growth-arresting effect of Bestatin is likely mediated through specific inhibition of LAP activity. The results point to the potential use of LAP as a drug target to develop novel anti-H. pylori agents, and Bestatin may provide a good lead for this purpose as it is well tolerated in vivo (Sakakibara et al., 1983).

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