INHIBITION OF *HELICOBACTER PYLORI* UREASE ACTIVITY *IN VIVO* BY THE SYNTHETIC NICKEL BINDING PROTEIN Hpn

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Helicobacter pylori infection is the most common cause of gastroduodenal ulcerations worldwide. Adaptation of *H. pylori* to the acidic environment is mediated by urease splitting urea into carbon dioxide and ammonia. Whereas neutralization of acid by ammonia is essential for gastric *H. pylori* colonization, the catalytic activity of urease is mediated by nickel ions. Therefore, nickel uptake and metabolism play key roles in *H. pylori* infection and urease is considered first line target for drug development and vaccination. Since nickel binding within *H. pylori* cells is mediated by the Histidine-rich protein designated Hpn, we investigated whether nickel binding by a synthetic Hpn is capable of abrogating urease activity of live *H. pylori* in liquid cultures. Supplementation of growth media with synthetic Hpn completely inhibited urease activity in live cells, indicating that *H. pylori* nickel uptake is effectively blocked by Hpn. Thus, nickel chelation by Hpn is stronger than nickel uptake of *H. pylori* offering therapeutic use of Hpn. Although the nickel binding of Hpn was confirmed by binding assays *in vitro*, its use in anti-*H. pylori* directed strategy will further need to be adapted to the gastric environment given that protons interfere with nickel binding and Hpn is degraded by pepsin.

Keywords: *Helicobacter pylori*, chronic gastritis, urease, nickel, histidine-rich protein

Introduction

The adaptation of the stomach pathogen *Helicobacter* (*H.*) *pylori* to the gastric environment depends on its ability to neutralize acid by ammonia ions generated from urea by the urease enzyme [1–6, see 4–6 for review]. Therefore, *H. pylori* expresses large amounts of urease and levels can reach up to 10% of total cellular protein. Native *H. pylori* urease is a multimeric protein that consists of six UreA and six UreB subunits forming the apoenzyme. The apoenzyme itself is not active without nickel ions, which are actively inserted in the active sites of each UreB subunit by nickel chaperone proteins [4–6]. Another important *H. pylori* nickel enzyme is a hydrogen uptake NiFe hydrogenase which is involved in electron transfer and respiration [5]. The essential role of nickel for *H. pylori* is further underlined by the strict regulation of nickel metabolism in the

bacteria [7]. In consequence, *H. pylori* has a high demand for nickel and the essential role of urease in gastric colonization renders the protein a top target for drug development or vaccination against *H. pylori* infection [6].

On the other hand, these findings indicate that nickel metabolism represents another target for alternative therapies against *H. pylori* infection. The binding of nickel in the cytoplasm of *H. pylori* is mediated by the small (7 kDa) protein Hpn (Hp1427), which was initially isolated through its binding to nickel *in vitro* [8]. The 60 amino acids of Hpn contain 28 Histidines and 4 Cysteines mediating nickel binding. In line with its nickel storing or scavenging function, *H. pylori* hpn mutants are sensitive to nickel overload [9]. These mutants were not affected in their urease activity, indicating that Hpn is not required for nickel transport or urease apo-protein activation [8]. Although Hpn was shown to form complexes

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with zinc *in vitro*, resistance to zinc was not affected in the *H. pylori* hpn mutant [8–10]. In order to investigate if nickel binding by Hpn could be of use to inhibit urease activity of *H. pylori* *in vivo*, we produced a synthetic version of the Hpn protein.

Materials and methods

Synthesis of H. pylori Hpn

The synthesis of the 60 mere peptide was performed on an ABI 433A automated peptide synthesizer on a 0.1-mM scale with 100 mg H-Rink-ChemMatrix resin (capacity 0.5 mmol/g), using double coupling and the Fmoc/tBu strategy. The following side-chain protecting groups were used: t-butyl ether (Ser, Thr, Tyr), t-butyl ester (Asp, Glu), and trityl (Gln and His). Couplings were performed with N-[1H-benzotriazol(1-yl)](dimethylamino)-methylene]-N-methylmethanaminium hexafluorophosphate-N-oxide (HBTU) in N-methylpyrrolidone as coupling agent. Deprotection of the Fmoc group was performed during the complete synthesis with 20% piperidine in DMF. The final deprotection from the resin was performed with 95% TFA in water containing 3% triisopropylsilane and 5% phenol. The crude peptide was purified by reverse phase high-performance liquid chromatography (RP-HPLC) on a VYDAC C18 column (40 × 300 mm, 1520 μ, 300 Å) with a linear gradient of 20% A to 60% B in 45 min (A: 1000 ml water, 2 ml TFA; B: 500 ml acetonitrile, 100 ml water, 1 ml TFA) at a flow rate of 70 ml/min with spectrophotometric monitoring at λ = 220 nm. The fractions were checked by RP-HPLC (Shimadzu LC10) on a Zorbax column with a linear gradient of 10 to 100% B over 45 min to give the final pure product. The mass spectrum was obtained using a ABI Voyager De Pro system. The correct mass of HpN is 7076 g/mol.

Nickel binding of synthetic H. pylori Hpn

The nickel binding capacity of Hpn was analyzed by co-incubation of 2 nmol Hpn with nickel agarose (Qiagen) at increasing concentrations (0, 2, 4, 6, and 8 μl) in phosphate buffered solution with a total volume of 30 μl. After 1 h incubation at 4 °C under shaking conditions, nickel agarose was removed by centrifugation (10 min, 4 °C, 13,000 rpm) and 20 μl of supernatant were added on a denaturing sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Afterwards removal of Hpn by the nickel agarose was confirmed by silver staining.

Aggregation of Hpn after preincubation with nickel and other metal ions

Synthetic Hpn (50 μM in a total reaction volume of 20 μl) was co-incubated with nickel or other metal ions

(500 μM). After incubation for 1 h at room temperature, samples were separated by native PAGE under non-denaturing conditions and proteins were visualized by silver staining. As indicated, the nickel chelator glyoxime was added to binding reactions at concentrations of 500 μM or 50 μM.

Results and discussion

Synthesis, structural integrity, and nickel binding of synthetic H. pylori Hpn

The 60 amino acid sequence of the Hpn protein was deduced from the genome sequence of *H. pylori* strain 26695 (Fig. 1). The 7 kDa protein was synthesized at the Institute of Biochemistry, Charité – University Medicine Berlin (Berlin, Germany) as described in Materials and methods.

Correct structural integrity of synthetic Hpn was confirmed by HPLC, mass spectrometry (Fig. 1), denaturing SDS polyacrylamide gel electrophoresis and silver staining (PAGE, Fig. 2). The nickel binding capacity of Hpn described earlier [10–12] was analyzed by co-incubation of 2 nmol Hpn with nickel agarose at increasing concentrations in phosphate buffered solution with a total volume of 30 μl. After 1 h incubation at 4 °C under shaking conditions, nickel agarose was removed by centrifugation (10 min, 4 °C, 13,000 rpm). Supernatants (20 μl) were analyzed by denaturing SDS-PAGE, and the protein was visualized by silver staining. Hpn was completely removed from the supernatants of binding reactions by the nickel agarose (Fig. 2).

Nickel binding and aggregation of synthetic Hpn

In order to further investigate the metal binding properties of synthetic Hpn, the protein was co-incubated with nickel or other metal ions (Fig. 3) for 1 h at room temperature and separated by native PAGE under non-denaturing conditions. Visualization by silver staining revealed that two states of Hpn were separated by native PAGE. The monomeric form HpnA migrated faster than the aggregated form HpnB (Fig. 3). In the presence of nickel, the monomeric HpnA form was completely transformed into the aggregated HpnB form. By comparing the migration to bovine serum albumin as a marker, it can be estimated that HpnB aggregates consist of 5–6 HpnA monomers (Fig. 3). This behavior confirmed the earlier finding that nickel binding stabilizes the aggregated form of Hpn [11, 12]. Notably, HpnA monomers were not transformed to HpnB in the presence of sodium and other cations, indicating that the metal interaction of Hpn is specific for nickel. The nickel chelator glyoxime at concentrations of 500 μM but not 50 μM inhibited transformation of HpnA monomers to HpnB, indicating that the nickel binding capacity of Hpn is similar or even stronger as compared to glyoxime (Fig. 3).

Fig. 1. Purification and quality control of the synthetic Hpn protein. The 60 amino acids protein sequence deduced from the genome sequence of *H. pylori* strain 26695 (A) was synthesized, purified by HPLC (B) and correct size of 7 kDa was confirmed by mass spectroscopy analysis (C)

Fig. 2. Binding of synthetic Hpn to nickel. Denaturing SDS-PAGE of synthetic Hpn in supernatants of binding reactions after pre-incubation with 0, 2, 4, 6, and 8 μ l nickel agarose as indicated above the lanes. Binding of Hpn to nickel was confirmed by complete removal of Hpn from supernatants of binding reactions with 2–8 μ l nickel agarose

Inhibition of urease activity by synthetic Hpn

To investigate if nickel binding by synthetic Hpn can abrogate urease activity in live bacteria, we cultivated *H. pylori* strain 26695 in Brucella Broth with 10% fecal calf serum (BBF) supplemented with nickel at a concentration of 10 μ M. Urease activity was measured in lysates from bac-

Fig. 3. Aggregation of Hpn is induced by nickel but not by other cations. Native PAGE of *H. pylori* Hpn (50 μ M each) after pre-incubation with nickel (Ni) or other cations (50 μ M each for 1 h at room temperature, as indicated above the lanes). HpnA, non-aggregated Hpn; HpnB, aggregated Hpn (the size of 40 kDa is estimated by comparing with the migration of the marker protein; M, marker protein (bovine serum albumin, 66 kDa); Na, sodium; Co, cobalt; Fe, iron; Ca, calcium; Zn, zinc; Cu, copper; +, Glyoxime 50 μ M; ++, Glyoxime 500 μ M. Nickel induced the formation of HpnB. Glyoxime at concentrations of 500 μ M but not 50 μ M inhibited nickel binding and formation of HpnB

teria harvested by centrifugation as described earlier [13] with the Berthelot reaction (Fig. 4). In this medium, urease activity was completely dependent on nickel supplementation and not measurable in bacteria grown in BBF without nickel (not shown). Most importantly, presence of 10 μ M synthetic Hpn in the BBF media did completely inhibit urease activity of live bacteria, indicating that *H. pylori* nickel uptake is effectively blocked by synthetic

Fig. 4. Inhibition of urease activity by synthetic Hpn added to *H. pylori* in liquid cultures. Bacteria cultivated for 48 h in liquid BBF with 10 μ M nickel were harvested by centrifugation. Urease activity expressed in Units (U) on the y axis was determined in lysed bacteria with the Berthelot reaction as described earlier. Supplementation of BBF with Hpn or glyoxime (10 μ M each) is indicated on the x axis (+/-)

Hpn (Fig. 4). Urease activity was also completely absent in control cells from cultures supplemented with the nickel chelator glyoxime at a concentration of 10 μ M. Neither glyoxime nor Hpn inhibited growth of bacteria.

Taken together with earlier findings, these results indicate that the synthetic Hpn protein displays a strong nickel binding activity and is suited to inhibit urease activity of live *H. pylori*. This provides strong evidence that nickel chelation by Hpn is stronger than nickel uptake of *H. pylori*. However, a therapeutic use of Hpn or another nickel scavenger is put in perspective by the fact that protons interfere with nickel binding and Hpn is degraded by gastric pepsin. Thus, use of nickel scavenging as a possible therapeutic strategy needs to be adapted to the gastric environment.

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

S.B., K.A.H., P.H., and M.M.H. conceived and designed the experiments. A.F. and K.A.H. performed the experiments. P.H. produced synthetic Hpn. S.B., A.F., K.A.H., P.H., U.B.G., and M.M.H. analyzed the data. S.B., A.F., M.M.H., and K.A.H. wrote the paper.

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