Helicobacter pylori catalase

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Helicobacter pylori is the major aetiological agent of gastroduodenitis in humans. Due to the potential importance of catalase in the growth and survival of *Helicobacter pylori* on the surface of inflamed mucosae, we have characterized catalase from *H. pylori* as a prelude to further studies on the function of the enzyme *in vivo*. The catalase activity of *H. pylori* was significantly affected by the presence of blood, serum or erythrocytes in the growth medium: the greatest activity was expressed when the bacterium was grown on medium containing serum. *H. pylori* catalase is a tetramer with a subunit M_r of 50 000. The enzyme had a pI of 9.0–9.3, was active over a broad pH range and was stable at 56 °C. It was non-competitively inhibited by sodium azide, and had no detectable peroxidase activity. The K_m for the purified catalase was measured as 43 ± 3 mM- H_2O_2 and the V as 60 ± 3 mmol H_2O_2 min⁻¹ (mg protein)⁻¹. The native catalase has absorption maxima at 280 nm and 405 nm with further minor shoulders or peaks at 510 nm, 535 nm and 625 nm, consistent with the presence of an iron-porphyrin prosthetic group.

Introduction

Catalase (EC 1.11.1.6) is an important enzyme for the protection of bacteria against the damaging effects of hydrogen peroxide. Although catalase activity does not always correlate with resistance to exogenous H_2O_2 , this enzyme is important to some bacteria in resisting the effects of toxic oxygen species released during inflammatory processes, particularly those involving polymorphonuclear leucocytes (Schwartz *et al.*, 1983; Beaman *et al.*, 1985; Wilson & Weaver, 1985).

Helicobacter (*Campylobacter*) *pylori* is the pathogen primarily responsible for gastroduodenitis in humans (Goodwin *et al.*, 1986; Hazell *et al.*, 1986a, b). In adults *H. pylori* gastritis begins with an acute inflammatory response (predominantly polymorphonuclear leucocytes) followed by infiltration with mononuclear cells. This results in the characteristic inflammatory lesion associated with *H. pylori* infection, acute or chronic gastritis (Morris & Nicholson, 1987; Frommer *et al.*, 1988) which persists despite the cellular and humoral response to the presence of the organism (Jones *et al.*, 1984). The mechanisms whereby *H. pylori* is able to thrive in such an apparently hostile environment remain to be elucidated. Because of the potential role played by catalase in maintaining a stable environment for the growth of *H. pylori in vivo*, we have characterized the enzyme as a prelude to a better understanding of its role as a putative

virulence factor of *H. pylori*. Part of this work was presented as a poster at the Vth International Campylobacter Workshop, Puerto Vallarta, Mexico, 1989.

Methods

Bacteria. *Helicobacter pylori* strains were isolated from gastric biopsies obtained from patients undergoing endoscopy at the Veterans' Affairs Medical Center, Houston, Texas. Strains were characterized as previously described (Hazell *et al.*, 1986b). *Campylobacter jejuni* strains were obtained from the hospital microbiology department culture collection. *H. pylori* isolates were designated 8826 (laboratory-adapted isolate), 8801, 8802, 8803 and 8804 (fresh isolates).

Culture conditions. Cultures were incubated at 37 °C in an environment of 10% (v/v) CO_2 in air and 99% relative humidity. Unless otherwise specified, cultures were grown on 5–7% (v/v) horse blood agar. When testing for the effect of blood products on catalase activity *H. pylori* was grown on a semi-defined basal medium [Iso-Sensitest Agar (ISA), Oxoid] and ISA supplemented with either 5% citrated human blood (blood agar), 5% human serum (ISA/serum) or 5% human erythrocytes washed in Alsevier's solution (ISA/RBC) as previously described (Hazell *et al.*, 1989). The blood or blood products were obtained fresh from a healthy *H. pylori*-negative volunteer.

Catalase extraction and chromatography. *H. pylori* cells were washed from agar in 0.1 M-potassium phosphate buffer, pH 7.35, containing 0.005% merthiolate (Eli Lilly & Co) (buffer A) and 1.0 µg ml⁻¹ leupeptin (Boehringer) (buffer A-L). The cells were centrifuged at 17 000 g for 8 min, resuspended in buffer A-L and disrupted by three cycles through a French pressure cell. This extract was then centrifuged at 17 000 g for 8 min, and the supernatant was recovered and recentrifuged at 75 000 g for 45 min. In the case of isolate 8826, due to

the presence of excess insoluble material that interfered with the chromatography, the final supernatant was mixed 50:50 with Percoll (Pharmacia) and centrifuged at 39000 g for 20 min. Insoluble material was discarded.

The final supernatant or the final supernatant in Percoll was applied to a K 26/100 gel filtration column of Sephacryl S-300 HR (Pharmacia) using buffer A as the liquid phase at a flow rate of 0.5 ml min⁻¹ and eluted. The catalase-positive fractions were pooled and concentrated 10-fold (Amicon Centriprep 30 concentrator, W. R. Grace & Co., Danvers, MA, USA). The concentrate was brought back to the original volume with deionized distilled water. The crude catalase was filtered (0.2 μ m pore size membrane), and then applied to a Domnick Hunter (now Millipore) MEMSEP 1000-CM ion-exchange capsule (Phenomenex, Rancho Palos Verdes, CA, USA) previously equilibrated with 1/10 concentration buffer A. After washing to remove unbound proteins, the catalase was eluted with a linear gradient generated by 1 M-KCl in 1/10 buffer A. The catalase was then rechromatographed through the Sephacryl S-300 HR column. Merthiolate was removed by rapid dialysis against 0.1 M-phosphate-buffered saline (PBS), pH 7.35, using the Amicon Centriprep 30 concentrator. The merthiolate-free catalase was filter-sterilized (0.2 μ m pore size membrane) and stored in the dark at 4 °C.

Determination of catalase activity. Qualitative catalase activity was tested by adding 50 μ l of the test material to 100 μ l of a 3% (v/v) H₂O₂ solution in a microtitre plate and watching for the rapid evolution of oxygen.

Quantitative catalase activity was determined after the method of Beers & Sizer (1952). Solutions of 10–200 μ l of 30% (v/v) H₂O₂ in 50 ml 0.1 M-PBS, pH 7.35 at 25 °C were prepared and the concentration of each solution determined spectrophotometrically using a molar absorption coefficient of 43.481 mol⁻¹ cm⁻¹ at 240 nm. Reaction kinetics of isolated catalase were determined by mixing 100 μ l enzyme in 2.9 ml H₂O₂ solutions with the initial reaction rates recorded using a programmable printer (Spectrophotometer 252, Gilford Instrument Laboratories). All assays were repeated to give 12 rate determinations for the first minute of reaction and these were recorded as the rate of decomposition of H₂O₂ min⁻¹ (mg protein)⁻¹. K_m and V values were determined by Lineweaver–Burk plots. The line of best fit and regression analysis was computed using Microstat (Ecosoft Inc., Indianapolis, IN, USA).

Comparisons of the catalase activities of whole bacteria were made using 100 μ l of a cell suspension consisting of bacteria washed in ice-cold PBS and adjusted to OD₆₀₀ = 1.0. A standardized solution of H₂O₂ in PBS (A_{240} = 0.55) at 25 °C was used for these assays with the reaction rate determined as above. Data from experiments using whole cells were compared using analysis of variance.

Characterization of catalase. The M_r of the native catalase was determined by gel filtration through fresh Sephacryl S-300 HR in 0.1 M-potassium phosphate buffer, pH 7.35, without merthiolate – which can interact with some proteins in the column – as compared to gel filtration M_r markers (Sigma). The subunit M_r was determined by SDS-PAGE under fully denatured and reduced conditions using SDS M_r markers (Bio-Rad). The isoelectric point was measured on a PhastGel IEF polyacrylamide gel (Pharmacia). Proteins were stained with Coomassie brilliant blue R dissolved in methanol/acetic acid.

Peroxidase activity was assayed spectrophotometrically using 4-chloro-1-naphthol/H₂O₂, dissolved in 0.05 M-Tris/HCl, pH 7.5, containing 0.2 M-NaCl, as substrate. Purified enzyme (100 μ l) was mixed with 100 μ l of substrate solution in the well of a microtitre plate and the plate, with the inclusion of appropriate controls, was incubated for 30 min in the dark at room temperature. Colour development was measured on a Titertek Multiskan instrument (Flow Laboratories).

The pH optimum of *H. pylori* catalase was tested in 0.1 M-potassium phosphate over a pH range of 5.25–8.95. Heat lability of *H. pylori*

catalase in 0.1 M-PBS, pH 7.35, was tested by heating the enzyme in a water bath at 56 °C for 60 min. Aliquots were removed at 10 min intervals and their catalase activity measured quantitatively. The effect of sodium azide on catalase kinetics was tested at a concentration of 50 nM-sodium azide in the presence of 0.25 ng catalase. The reaction rate was determined as outlined above. Reversibility of azide inhibition was tested after dialysis of azide-treated catalase against 0.1 M-PBS, pH 7.35 (three changes of buffer over 18 h at 4 °C). The spectrophotometric profile of the catalase was determined by scanning both the native protein and enzyme treated with K₃Fe(CN)₆/KCN (Tentori & Salvati, 1981) in the range 260–680 nm.

Protein determination. Protein estimations were made by either the bicinchoninic acid (BCA) or the microBCA protein assay systems (Pierce Chemical Co.). Bovine serum albumin was the protein standard.

Results

Effect of blood and blood products on catalase expression by *H. pylori*

The whole-cell catalase activity of *H. pylori* isolate 8826 grown on ISA, blood agar, ISA/serum and ISA/RBC over 96 h demonstrated that the length of incubation and the type of blood product in the medium had a significant effect ($P < 0.001$) on the catalase activity (Fig. 1). This

Fig. 1. Effect of incubation period and medium supplements on (a) the yield of cells and (b) the expression of catalase by whole cells of *H. pylori* strain 8826 when grown on basal medium (ISA) (○) and basal medium containing human serum (ISA/serum) (□), blood (blood agar) (●) or erythrocytes (ISA/RBC) (■). Catalase activity is also shown for two strains of *C. jejuni*, 1186 (△) and 1284 (▲), grown on blood agar. The results in (b) are plotted as means $\pm$ 95% confidence intervals.

medium effect was also apparent with four other isolates (8801, 8802, 8803 and 8804) as determined using whole cells following 3 d incubation (Fig. 2). The whole-cell catalase activity of two strains of *C. jejuni* tested concurrently was about five times less active than *H. pylori* ($P < 0.001$) at the same specified H_2O_2 concentration (Fig. 1).

Characterization of catalase

H. pylori catalase was purified from two isolates (8826 and 8801) to the order of 65-fold. The catalase from both isolates had identical properties; representative data from isolate 8826 are given below. The native catalase had an apparent M_r of 190 000–200 000 as determined by gel filtration, whereas the reduced denatured enzyme

had an apparent M_r of the order of 50 000. The isoelectric point was in the range 9.0–9.3. The *H. pylori* catalase was active over a broad pH range, showing no significant difference in activity between pH 6.5 and 8.95. It was stable at 56 °C for 1 h and was reversibly inhibited by sodium azide. Kinetic data were consistent with this inhibition being non-competitive. No peroxidase activity was detected.

The kinetic properties of the catalase from *H. pylori* were determined; the K_m was measured as 43 ± 3 mM- H_2O_2 and the V as 60 ± 3 mmol H_2O_2 decomposed min^{-1} (mg protein) $^{-1}$.

The native catalase has absorption maxima at 280 nm and 405 nm (Soret band) and further minor shoulders or peaks at 510 nm, 535 nm and 625 nm, and a 405/280 ratio of 0.65. Treatment with $K_3Fe(CN)_6/KCN$ produced a spectral shift of the 405 nm peak to 422 nm (Fig. 3).

Fig. 2. Effect of medium supplements on whole-cell catalase expression by fresh isolates of *H. pylori*, 8801, 8802, 8803 and 8804, when grown on the basal medium (ISA) (▨), and basal medium containing human serum (ISA/serum) (■), blood (blood agar) (▤) or erythrocytes (ISA/RBC) (□). Cultures were incubated for 3 d. The results are means $\pm$ 95% confidence intervals.

Fig. 3. Shift in the absorption spectrum of the native *H. pylori* catalase following treatment with $K_3Fe(CN)_6/KCN$.

Discussion

Catalase is a ubiquitous enzyme found in eukaryotic and most prokaryotic organisms. The native *H. pylori* catalase appears to be a tetramer (M_r 200 000) and the spectral analysis reported here is consistent with the presence of an iron-porphyrin prosthetic group (Hochman & Shemesh, 1987). The enzyme is soluble and may be distributed throughout the cytosol and in the periplasmic space (unpublished results). Whether the catalase is loosely associated with specific membrane proteins remains to be determined.

H. pylori catalase was reversibly and non-competitively inhibited by sodium azide. Azide combines reversibly with iron-porphyrin prosthetic groups and causes a reduction of the iron to the ferrous state (Keilin & Hartree, 1945; Lamberg & Foulkes, 1948). Azide and cyanide may appear as non-competitive catalase inhibitors; however, under conditions of high substrate concentration they may act as competitive inhibitors (Ogura, 1955). The enzyme was heat stable and had a broad pH activity similar to that seen with *Klebsiella pneumoniae* (KpT) and *Escherichia coli* (HP11), the latter having two distinct activity peaks at pH 6.8 and 10.5 (Meir & Yagil, 1985; Goldberg & Hochman, 1989). Unlike the *E. coli* catalases (HP11 and HP1; Meir & Yagil, 1985), no concomitant peroxidase activity was detected for *H. pylori* catalase. Heat stability is a property shared by the non-peroxidase catalases (i.e. the 'typical' catalases) and the characteristics of *H. pylori* catalase are consistent with the enzyme being a 'typical' catalase.

The isoelectric point of the *H. pylori* catalase (9.0–9.3) appears unique. The isoelectric point of catalase usually occurs at an acid pH (e.g. *Rhodospseudomonas capsulata*,

4.5, and *Pseudomonas pyrocinia*, 5.0; Weisner *et al.*, 1985; Hochman & Shemesh, 1987). This characteristic of the enzyme may represent an adaptation to a unique environment.

In media supplemented with different blood products, there was a fall in the catalase activity of *H. pylori* as the relative concentration of erythrocytes increased and that of serum decreased. This is consistent with either a serum factor(s) stimulating catalase activity and/or the presence of haem-containing proteins, released from erythrocytes by the action of *H. pylori* (Wetherall & Johnson, 1989), suppressing the synthesis of catalase. Suppression of synthesis of porphyrins by haem groups has been demonstrated in mature mammalian cells; and in rodents, liver catalase activity increases subsequent to serum injection (Weil-Malherbe & Schade, 1948; Woods, 1974). In *E. coli*, the expression of catalase activity is subject to catabolite repression in the presence of glucose (Meir & Yagil, 1990). Whether glucose, or other compounds, can repress the catalase activity of *H. pylori*, which is non-glycolytic, remains to be determined. Also, unlike *E. coli* (Meir & Yagil, 1990), the expression of catalase in *H. pylori* was not enhanced by the cells entering stationary phase, as the highest activity occurred in young cultures. Two or possibly three catalases have been identified in *E. coli* (Meir & Yagil, 1985; Peyru & Fraenkel, 1968). In the studies reported here we have identified one isomer of catalase. Whether other isomers of catalase can be induced in *H. pylori* under conditions such as oxidative stress remains to be determined.

The catalase activity of *H. pylori* was significantly greater than that of the related bacterium *C. jejuni*. *H. pylori* is a major aetiological agent of human acute on chronic gastritis and a pronounced catalase activity may aid the survival of the bacterium *in vivo*. Beaman *et al.* (1985) noted that resistance of *Nocardia asteroides* to the microbicidal activity of polymorphonuclear leucocytes, which can release H_2O_2 both intra- and extracellularly (Clifford & Repine, 1982), was mediated by both superoxide dismutase and catalase. However, the presence or absence of catalase does not render absolutely a bacterium more or less susceptible to the lethal effects of H_2O_2 , as susceptibility to H_2O_2 can also be dependent upon the sensitivity of cellular components to oxidative damage (Schwartz *et al.*, 1983; Wilson & Weaver, 1985). We (Hazell *et al.*, 1989; Hazell & Graham, 1990) have previously demonstrated that bovine liver catalase can aid the growth of *H. pylori in vitro*, this being due, in part, to the prevention of formation of toxic peroxidated fatty acids. These data would indicate that *H. pylori* is at least indirectly sensitive to oxidative damage. Whereas endogenous catalase of *H. pylori* does not appear to play a role in conditioning the external environment, it may be critical at the cellular level.

The K_m for *H. pylori* catalase was 3–10 times higher than that recorded for most other bacteria, but about one-third that of heat-stable mycobacterial catalase. Also, the enzyme was stable at concentrations of H_2O_2 known to inhibit other bacterial catalases, a property shared with several species of the genus *Mycobacteria* (Wayne & Diaz, 1982; Meir & Yagil, 1985; Allgood & Perry, 1986; Hochman & Shemesh, 1987; Goldberg & Hochman, 1989). These properties could relate to the adaptation of the mycobacterial and *H. pylori* enzymes to an environment rich in toxic oxygen species.

This paper provides fundamental information on the nature and activity of *H. pylori* catalase and is a prelude to further studies that will determine if this enzyme is important in enabling *H. pylori* to survive on the surface of inflamed gastroduodenal mucosae.

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