A *cb*-type cytochrome-*c* oxidase terminates the respiratory chain in *Helicobacter pylori*

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A *Helicobacter pylori* membrane fraction oxidized yeast and equine cytochrome *c*, and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). When ascorbate was used as reductant, the $V_{\max}$ and apparent K_m values were 612 nmol electron min⁻¹ (mg protein)⁻¹ and 14 μ M for yeast, and 419 nmol electron min⁻¹ (mg protein)⁻¹ and 19 μ M for equine cytochrome *c*, respectively. For TMPD oxidation, the $V_{\max}$ and K_m values were 640 nmol electron min⁻¹ (mg protein)⁻¹ and 182 μ M, respectively. These oxidase activities showed a high affinity for oxygen. Inhibition of both cytochrome-*c* and TMPD oxidase activities by 50% was caused by about 4 μ M cyanide and about 0.5 mM azide. Redox difference spectra of the membrane solubilized with Triton X-100 showed *b*- or *c*-type cytochromes but not *aa*₃-type cytochromes. *c*-type and a part of some *b*-type cytochromes were reduced with ascorbate plus TMPD. A CO difference spectrum revealed that protohaem, but not an *aa*₃-type cytochrome, may be interacting with CO/oxygen. Only protohaem was detected in the haem fraction extracted from the membrane. Three polypeptides (60, 38 and 29 kDa) were found to be bearing haem *c* after SDS-PAGE of the membrane. From these results, it was suggested that the *cbb*₃-type cytochrome-*c* oxidase, having a haem-copper binuclear centre like the cytochrome *aa*₃-type oxidase, but differing in a few other properties, functions as a terminal oxidase in the respiratory chain of *H. pylori*.

Keywords: *Helicobacter pylori*, microaerobic respiration, cytochrome-*c* oxidase

INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic, spiral bacterium that resides in the stomach of humans. This organism has been implicated as an essential factor or direct cause of gastritis, duodenal ulcers, and possibly neoplasia (Blaser, 1990; Marshall & Warren, 1984; Parsonnet *et al.*, 1991, 1994; Rabeneck & Ransohoff, 1991). *H. pylori* lives in the mucous layer overlying the gastric epithelium of humans, where the micro-environment is low in oxygen because of rapid growth of the epithelium cells. *H. pylori* is an obligate aerobe which does not grow in the presence of normal air-oxygen pressure nor anaerobically in the absence of oxygen. *H. pylori* does not catabolize saccharides (Marshall *et al.*, 1984; McNulty & Dent, 1987), but derives energy from

the metabolism of organic acids and amino acids via the Krebs cycle. *H. pylori* is oxidase positive and has been reported to have menaquinone-6 and an unidentified quinone as respiratory quinones (Goodwin *et al.*, 1986; Moss *et al.*, 1990). However, the biochemical properties of the respiratory and oxidase systems of *H. pylori* have not been characterized.

In aerobic eubacteria, the cytochrome *aa*₃ or *caa*₃-type cytochrome-*c* oxidase (EC 1.9.3.1) is very similar to the mitochondrial enzyme with respect to its chromophores and the main subunits (subunits I–III) (Buse *et al.*, 1989; Calhoun *et al.*, 1993; Saraste *et al.*, 1991; Sone & Yanagita, 1982). During the past several years, atypical cytochrome-*c* oxidases such as *cbb*₃, *ba*₃ and *bo*₃-types, as well as quinol-oxidizing terminal oxidases of the *aa*₃-type and *bo*₃ and *bd*-types, have been found. These oxidases, except for the *bd*-type, have been shown to possess a haem (high-spin)-copper (Cu_B) binuclear centre, although species of haem can differ (see Garcia-Horsman *et al.*, 1995 for review).

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Moreover, these oxidases have homologous amino acid sequences, at least in subunit I, which bears the oxygen-reducing binuclear centre and an electron-donating low-spin haem (Saraste & Castresana, 1994).

Among the haem-copper oxidase superfamily, the *cbb₃*-type cytochrome-*c* oxidases, found in *Rhodobacter capsulatus* (Gray *et al.*, 1994), *Rhodobacter sphaeroides* (Garcia-Horsman *et al.*, 1994) and *Bradyrhizobium japonicum* (Preisig *et al.*, 1993), seem to be a primitive group, closely related to NO reductase according to sequence similarity (Bott *et al.*, 1992; Keefe & Maier, 1993; Preisig *et al.*, 1993). *cbb₃*-type cytochrome oxidase appears to be produced as an adaptation to microaerobic conditions (Bott *et al.*, 1992; Garcia-Horsman *et al.*, 1994), and it contains subunits II and III with no homology to their counterparts in *aa₃*-type cytochrome-*c* oxidases (Preisig *et al.*, 1993). The terminal oxidases belonging to the haem-copper superfamily are known to be inhibited by cyanide at a sub-micromolar order and to have a very low K_m for oxygen of a micromolar order (Garcia-Horsman *et al.*, 1995). Here we report the characteristics of cytochrome-*c* oxidase activity and the absorption spectra of the membrane fraction of *H. pylori* grown under microaerobic conditions.

METHODS

Reagents. *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (TMPD), trifluoroacetic acid, *o*-tolidine and $\text{Na}_2\text{S}_2\text{O}_4$ were from Wako Pure Chemical. NADH, equine heart and yeast (*Saccharomyces cerevisiae*) cytochrome *c*, duroquinone and tetrachloroquinone were purchased from Sigma. Menaquinone-4 and ubiquinone-1 were gifts from Eisai. Thermophilic bacterial PS3 cytochrome-*c* oxidase was purified as described previously (Sone & Yanagita, 1982). Beef-heart cytochrome-*c* oxidase (Orii *et al.*, 1977) and *Escherichia coli* *bo₃*-type quinol oxidase (Tsubaki *et al.*, 1993) were kindly donated by Dr Y. Orii of Kyoto University, Japan, and by Dr T. Mogi and Professor Y. Anraku of the University of Tokyo, Japan, respectively.

Bacterial strain, growth conditions and preparation of a membrane fraction. *H. pylori* NCTC 11637 was used throughout the present work and was cultured on Skirrow's medium plates containing 7% (v/v) defibrinated horse blood as described previously (Nagata *et al.*, 1992). The cultures were incubated for 3 d at 37 °C in a microaerophilic atmosphere [$\text{O}_2/\text{CO}_2/\text{N}_2$ (5:15:80, by vol.)]. The cultured cells were harvested by centrifugation (10000 *g* for 10 min at 4 °C), washed with PBS (0.139 M NaCl; 3 mM KCl; 10 mM sodium potassium phosphate) at pH 7.0, and stored as a cell pellet at -80 °C. A membrane fraction of *H. pylori* was prepared as follows. The cell pellet was suspended in a preparation medium containing 0.25 M sucrose, 10 mM KCl, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, 20 mM Tris/maleate buffer (pH 6.5) and 10 mM potassium phosphate buffer (pH 6.8), and the suspension was sonicated for 30 s four times on ice. After centrifugation (10000 *g*, 20 min, 4 °C) to remove cell debris, the supernatant was centrifuged (100000 *g*, 90 min, 4 °C), and the pellet was suspended in the preparation medium and used as the membrane fraction.

Assay of oxidase activity. Oxygen uptake was monitored with an oxygen electrode (YSI no. 4001) in a semi-closed cell. Various substrates were added to 2 ml of reaction medium containing 10 mM KCl, 2 mM MOPS buffer (pH 6.8) and the

membrane fraction (0.1–1.0 mg protein). When quinols were used, DTT (final concentration 2 mM) was added to the reaction medium. Each quinone was reduced with DTT in the medium for 5 min, and the membrane fraction was added to start the reaction. The apparent activity due to autooxidation of each quinol and DTT was subtracted. The K_m for oxygen was determined by recording traces of oxygen uptake in the medium with a very low concentration range of oxygen using a high-sensitivity strip chart recorder (Yokokawa model 3066) as described previously (Sone & Fujiwara, 1991a). The concentration dependence of cytochrome *c* and TMPD was measured using a glass electrode with ascorbate as a final electron donor according to the following equation, as described previously (Nicholls & Sone, 1984): sodium ascorbate + H^+ + $1/2 \text{O}_2 \rightarrow$ dehydroascorbate + H_2O + Na^+ . The net alkali formation was determined by back-titration with 10 mM HCl. The reaction medium (2 ml) comprised 10 mM KCl, 10 mM sodium ascorbate and 2 mM MOPS/KOH (pH 6.8).

Determination of concentrations giving 50% inhibition (IC_{50}). Inhibition of cytochrome-*c* and TMPD oxidase activities by KCN and azide was assayed using a Beckman DU-700 spectrophotometer. The oxidation of yeast ferrocyanochrome *c* and TMPD was followed at 550 and 610 nm, respectively. The reaction was initiated by adding 0.01 ml of the membrane fraction to 0.5 ml of the reaction medium containing 10 mM MOPS/KOH buffer and an appropriate amount of reduced cytochrome *c* or TMPD in a cuvette of 1 cm light path. Percentage inhibition was determined by the following equation: inhibition (%) = [(activity without inhibitors – activity with inhibitors) / (activity without inhibitors)] $\times$ 100. IC_{50} is the concentration of KCN or azide which gave 50% inhibition.

Measurement of absorption spectra. Redox difference and CO difference spectra were recorded with a Beckman DU-70 spectrophotometer using a 1 cm light-path microcuvette. The sample of solubilized membrane fraction was prepared as follows: membrane fraction (10–20 mg protein ml^{-1}) was solubilized in 2% Triton X-100, 0.4 M NaCl and 0.1 M Tris/HCl (pH 8.0) with a brief sonication and successive centrifugation at 10000 *g* for 10 min. The supernatant was used for spectrophotometry. The pelleted materials were scarce, and did not show cytochrome colour. Amounts of cytochrome *b* and *c* were estimated from the redox difference spectrum using ϵ_{mM} (563–577 nm) of 14.3 and ϵ_{mM} (550–535 nm) of 21.0, respectively (Williams, 1964). Dipyridine ferrohaemochrome analysis of the cytochromes was carried out according to the method of Berry & Trumpower (1987).

Analysis of haem. Non-covalently bound haem groups were extracted with HCl/acetone (Weinstein & Beale, 1983) and concentrated in ethyl acetate after several phase separations with 0.1 M NaCl. The extracted haem groups were analysed by reverse-phase chromatography with a 1 ml resource TM PRC column (Pharmacia) and monitored with a Waters 490 multiwavelength photometer. The solvents were water and acetonitrile, both containing trifluoroacetic acid (0.05%), and a gradient elution (30–100%) was done as described previously (Sone & Fujiwara, 1991b). Protohaem, haem *a* and haem *o* (with protohaem) were prepared from haemoglobin, beef-heart cytochrome-*c* oxidase and *E. coli bo₃*-type quinol oxidase, respectively, and used as standards.

Other methods. SDS-PAGE was carried out according to the method of Laemmli (1970). Staining of gels for haem-dependent peroxidase was carried out with *o*-tolidine (Reid & Ingledew, 1980). The amount of protein in samples was determined by a modified Lowry method (Markwell *et al.*, 1981).

RESULTS

Cytochrome-c oxidase activity

Table 1 gives typical *H. pylori* membrane fraction oxidase activity measurements with various saturating concentrations of substrate. The membrane fraction rapidly oxidized TMPD as well as cytochromes *c* from yeast and equine heart. The membrane fraction oxidized succinate, malate, lactate and NADH, showing the presence of the respective membrane-bound dehydrogenases. The rate of oxidation of these substrates was slow by comparison with oxidase activity. More than 90% of NADH, succinate and malate oxidation was inhibited by antimycin A (50 μ M). Several quinols were also oxidized, but much more slowly. These results indicated the presence of a cytochrome-*c* oxidase, rather than a quinol oxidase, as the dominant terminal oxidase in *H. pylori*.

Table 2 summarizes the kinetic parameters of *H. pylori* cytochrome oxidase. Yeast cytochrome *c* was a better

Fig. 1. Trace of TMPD oxidase activity of the membrane fraction of *H. pylori* (b) at a low oxygen concentration range compared with thermophilic bacterial PS3 cytochrome-*c* oxidase (a). About 50 μ M TMPD in the presence of 5 mM ascorbate was used as an electron donor, and the other assay conditions were described in Methods.

Table 1. Oxidase activity in the *H. pylori* membrane fraction with various electron donors

Substrate	Concn (mM)	Oxygen uptake [nmol min ⁻¹ (mg protein) ⁻¹]
Yeast cytochrome <i>c</i> *	0.045	294.9
Equine cytochrome <i>c</i> *	0.045	264.0
TMPD*	0.750	313.3
NADH	0.5	14.5
NADH with cytochrome <i>c</i> (40 μ M)	0.5	14.5
Malate	2.0	2.7
Succinate	2.0	5.7
Lactate	2.0	1.9
Menaquinol-4	0.2	6.2
Ubiquinol-1	0.2	3.3
Tetrachlorohydroquinol	0.15	8.3
Duroquinol	7.5	6.5

* In the presence of sodium ascorbate (final concentration 10 mM).

substrate than equine cytochrome *c*. The concentration dependencies of the oxidase for these cytochromes *c* showed sigmoidal-like curves (not shown). The activities at assumed real concentrations, with the adsorbed amounts subtracted (6 and 3 μ M for yeast and equine heart cytochrome *c*, respectively), followed Michaelis-Menten kinetics, and gave K_m values of 8 μ M for yeast cytochrome *c* and 16 μ M for equine cytochrome *c*. TMPD was also oxidized rapidly without showing 'adsorption'. Both cytochrome-*c* and TMPD oxidase activities were sensitive to inhibition by KCN and azide (Table 2).

We also measured the K_m for oxygen by following rates of oxygen uptake at a very low concentration range of oxygen with an oxygen electrode (Fig. 1). The K_m of the oxidase for oxygen was about 0.4 μ M. The value is a little lower than that of the *aa₃*-type (0.6 μ M). The pH dependency of cytochrome-*c* oxidase activity and its pattern was very similar to that of the mammalian

Table 2. Summary of oxidase activity kinetic parameters and its inhibition by KCN and azide in the *H. pylori* membrane fraction

	Substrate		
	Yeast cytochrome <i>c</i>	Equine cytochrome <i>c</i>	TMPD
V_{max} *	612	419	640
Apparent K_m (μ M)	14	19	182
IC ₅₀ (μ M) of KCN	4	—	2.5
IC ₅₀ (μ M) of azide	407	—	488

* nmol min⁻¹ (mg protein)⁻¹.

Fig. 2. Redox (a) and CO (b) difference spectra of the solubilized membrane fraction of *H. pylori*. The membrane (12.8 mg ml^{-1}) was solubilized as described in Methods. (a) —, Ascorbate (TMPD)-reduced minus oxidized; —, $\text{Na}_2\text{S}_2\text{O}_4$ -reduced minus oxidized. (b) $\text{Na}_2\text{S}_2\text{O}_4$ -reduced plus CO minus $\text{Na}_2\text{S}_2\text{O}_4$ -reduced. For complete reduction, a very small grain of $\text{Na}_2\text{S}_2\text{O}_4$ was added in the presence of 5 mM ascorbate and TMPD (below $10 \mu\text{M}$).

mitochondrial enzyme (Orii & Okunuki, 1965); the optimal pH was 5.6–6.0, and moderate activities were observed at pH 7–8 (not shown).

Visible spectral properties

Fig. 2(a) shows a reduced minus oxidized difference spectrum of a solubilized membrane fraction of *H. pylori*. Almost no absorption band was observed at around 600 nm, indicating that cytochrome aa_3 is absent or is only a very minor component. Both *b*-type and *c*-type cytochromes were prominent upon reduction with $\text{Na}_2\text{S}_2\text{O}_4$ (continuous line), but only *c*-type cytochromes and a part of some *b*-type cytochromes could be reduced with ascorbate plus TMPD (dotted line). From the redox difference spectra, the cytochrome *c* and *b* content was calculated to be 0.2 and 0.5 nmol per mg of *H. pylori* membrane fraction protein, respectively. These cytochrome levels in *H. pylori* are the same order as the levels found in many aerobes.

The CO difference spectrum (Fig. 2b) shows that the

Fig. 3. Elution profiles of haem compounds after reverse-phase HPLC. (a) Haem fraction extracted from the *H. pylori* membrane fraction. (b) Haem fraction from *E. coli bo3*-type quinol oxidase containing protohaem and haem *o*. (c) Haem fraction from beef heart cytochrome-*c* oxidase containing haem *a*.

trough due to the γ band of the reduced form is at 433 nm, indicating that the CO-binding cytochrome is not cytochrome aa_{33} , but is of the *b*-type or *o*-type. A very similar difference spectrum was reported for CO-reactive cytochrome *o* (Puustinen & Wikström, 1991; Sone & Fujiwara, 1991a).

Haem analysis

Haem *o* (2-hydroxyethylfarnesyl protohaem) constitutes the binuclear oxygen-reducing site as the high-spin haem in *E. coli bo*-type quinol oxidase (Puustinen & Wikström, 1991) and *Bacillus* cytochrome-*c* oxidase cultured under microaerobic conditions (Sone & Fujiwara, 1991a). The hydrophobicity of haem *o* is similar to haem *a*, but its spectrum is similar to protohaem. In order to distinguish between haem *o* and protohaem, the haem fraction was extracted from the *H. pylori* membrane preparation and analysed as shown in Fig. 3. Almost all the extracted haem was protohaem, and neither haem *o* nor haem *a* was present. Dipyridine ferrohaemochrome analysis of the extracted haem showed an α band at 557 nm to be the authentic protohaem (not shown).

c-type cytochromes have covalently bound haem *c*, and thus their distribution among the subunits of proteins can be analysed by a haem-staining procedure after SDS-PAGE, by exploiting the intrinsic peroxidase activity of haem groups (Reid & Ingledew, 1980). Fig. 4 shows a typical haem stain of the *H. pylori* membrane fraction. Three peroxidase-positive bands of apparent molecular mass 60, 38 and 29 kDa were found in the *H. pylori*

Fig. 4. Identification of *c*-type cytochromes of *H. pylori* after SDS-PAGE. *H. pylori* membrane protein (43 µg) was separated by SDS-PAGE and stained for peroxidase activity as described in Methods. Bio-Rad prestained marker proteins (106, 75, 49, 32, 27 and 17 kDa) were used.

membrane. It is noteworthy that no small *c*-type cytochrome was found in the *H. pylori* membrane fraction. On the contrary, a *c*-type cytochrome having an α band at 553 nm was found in the soluble fraction of *H. pylori*.

DISCUSSION

The present data using a membrane fraction of *H. pylori* clarified several characteristics of the respiratory chain and terminal oxidase of *H. pylori*: (1) cytochrome components were restricted to *b*- and *c*-type; (2) CO difference spectra and analysis of haem components showed that protohaem is interacting with oxygen; (3) cyanide inhibited the cytochrome-*c* and TMPD oxidase activities at a very low concentration; (4) the K_m for oxygen was low (about 0.4 µM in the TMPD oxidation); (5) the terminal oxidase oxidized cytochrome *c* not quinol; (6) three kinds of *c*-type cytochromes (60 kDa, 38 kDa and 29 kDa by SDS-PAGE) were present in the *H. pylori* membrane fraction. These results strongly suggest that the terminal oxidase of *H. pylori* is a *cbb*₃-type cytochrome-*c* oxidase.

H. pylori is a strict microaerobe. The most suitable gaseous conditions for growth are 5–7% oxygen and 7–10% carbon dioxide in nitrogen. *H. pylori* lives in the mucous layer of the human stomach and can be associated with any part of the lumina of gastric-mucus-secreting cells but is most frequently sited in the 'grooves' at the junction of

individual epithelial cells (Steer, 1989). In the environment of these 'grooves', the oxygen tension seems to decrease because epithelial cells in the human stomach grow very rapidly with a generation time of about 1 d (Lipkin *et al.*, 1963). Thus, *H. pylori* grows to adapt to the microaerobic niche *in vivo*, and the *cbb*₃-type cytochrome-*c* oxidase with a high affinity for oxygen identified in the present study is well-suited for this environment. Bacteria of the genus *Campylobacter*, to which *H. pylori* formerly belonged, are also microaerophilic. Our preliminary results have shown that the terminal oxidase of *Campylobacter jejuni* is also a *cbb*₃-type cytochrome-*c* oxidase (authors unpublished observations). Cytochrome (*cbb*₃-type oxidases may be widely found among microaerobic bacteria or those grown under microaerobic conditions. It has been shown that the *cbb*₃-type oxidase is induced under microaerobic conditions and that this oxidase is essential for nitrogen-fixing bacteroids (Preisig *et al.*, 1993). Recently, this type of cytochrome-*c* oxidase was reported in the non-endosymbiotic photosynthetic bacteria *R. capsulatus* and *R. sphaeroides* when grown under microaerobic conditions (Garcia-Horsman *et al.*, 1994; Gray *et al.*, 1994).

SDS-PAGE analysis revealed that three *c*-type cytochromes with rather high apparent molecular masses (60, 38 and 29 kDa) were present in the *H. pylori* membrane fraction (Fig. 4). The *cbb*₃-type cytochrome oxidases from *R. capsulatus*, *R. sphaeroides*, *B. japonicum* and *Azorhizobium caulinodans* are reported to have two haem C subunits (Garcia-Horsman *et al.*, 1994; Gray *et al.*, 1994; Keefe & Maier, 1993; Mandon *et al.*, 1994). Thus, the three proteins with haem *c* in the *H. pylori* membrane fraction (Fig. 4) may be assignable to *c*-type cytochrome subunits of cytochrome *cbb*₃-type oxidase and cytochrome *bc*₁ complex. The molecular masses of two *c*-type cytochromes in *A. caulinodans* are reported to be 30.9 kDa (*fixP*) and 27.6 kDa (*fixO*) (Mandon *et al.*, 1994) and those in *R. sphaeroides* are estimated to be 35 and 29 kDa (Garcia-Horsman *et al.*, 1994). Usually, cytochrome *c*₁ has a molecular mass of 26–33 kDa, but a *Paracoccus denitrificans* cytochrome *c*₁ has a molecular mass of about 62 kDa (Berry & Trumpower, 1985). One of these *c*-type cytochromes may be a component of the cytochrome *bc*₁ complex, and the rest seem to constitute cytochrome *cbb*₃-type oxidase. Some of the amount of the total *c*-type cytochromes [0.2–0.3 nmol (mg membrane protein)⁻¹] was comparable to the amount of high-spin protohaem which may react with oxygen/CO. If the peak in the γ band of high-spin protohaem of reduced *cbb*₃ has an ϵ_{mM} of 150 at 433 nm, its concentration is calculated to be 0.096 nmol (mg protein)⁻¹ from the difference spectra (Fig. 2b). It is thus likely that single high-spin protohaem is reacting with CO, and two or three different *c*-type cytochromes constitute the terminal oxidase of *H. pylori*. Most of these *c*-type and *b*-type cytochromes may have high redox potentials and so can be reduced with ascorbate (Fig. 2a).

Compared to the high activities of cytochrome *c* or TMPD oxidation, NADH oxidase activity was very low (Table 1). NADH oxidase activity determined spectro-

photometrically and NADH-ferricyanide reductase activity in the *H. pylori* membrane fraction were also low (data not shown). The reason why the NADH dehydrogenase activity was so low in the membrane fraction is not known at present. Addition of cytochrome *c* did not accelerate NADH oxidation (Table 1). However, FAD-containing type II NADH dehydrogenases, but not the membrane-intrinsic H⁺-pumping complex I (type I) enzyme, may detach from the membrane upon preparation (Sone, 1990), and these peripheral enzymes may reduce menaquinones of *H. pylori*. It is possible that the *H. pylori* membrane preparation is right-side out and NADH may not cross the membrane, since the preparation showed appreciable oxidase activities for cytochrome *c* as well as membrane-permeable TMPD. However, bacterial membrane preparations derived by sonication seldom produce right-side out membrane vesicles, and in fact the addition of detergents, such as sucrose monolaurate and Triton X-100 (0.1% in the reaction medium) did not stimulate NADH oxidation.

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