**Flavodoxin-dependent pyruvate oxidation, acetate production and metronidazole reduction by *Helicobacter pylori***

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Introduction

*Helicobacter pylori* is one of the commonest human pathogens. It is a major cause of gastritis and is strongly associated with peptic ulcer disease\(^2\) and gastric cancer.\(^2\) The growing clinical significance of *H. pylori* infection necessitates effective therapeutic strategies for eradicating the bacteria. Triple therapy has been the most successful method for eradicating *H. pylori*,\(^2\) giving an eradication rate of 80–90%. However, increasing metronidazole resistance has limited the usefulness of such therapy.\(^6\)–\(^8\)

The activation of metronidazole is closely linked to the oxidation of pyruvate,\(^9\) but *H. pylori* pyruvate metabolism is poorly understood. Recently, it has been reported that the organism can, to a limited extent, use glucose via the Entner–Doudoroff pathway,\(^10,11\) or the pentose phosphate pathway,\(^12\) generating acetate and lactate, respectively. Nuclear magnetic resonance (NMR) spectroscopy studies indicate that these end-products are formed by oxidation of pyruvate.\(^10\) The bacterium can also use alanine, serine and \(\alpha\)-lactate,\(^13\) and these substrates can be converted to pyruvate enzymatically in *H. pylori*.\(^14\)–\(^16\) Pyruvate is an important intermediate in the physiology of the bacterium, being at the branchpoint of several metabolic routes. However, the specific pathways that convert pyruvate to catabolic end-products have been only partly elucidated.\(^17\)–\(^18\)

The nitro group of nitroimidazoles have to be reduced in order to kill susceptible bacteria. It has been proposed that, in typical anaerobes, this reduction is mediated by a pyruvate ferredoxin (flavodoxin) oxidoreductase complex.\(^9,19\) Metronidazole can be transformed into a bactericidal agent by the electrons generated from the oxidation of pyruvate according to the equation:

\[
\text{pyruvate + CoA} \rightarrow \text{acetyl-CoA} + \text{CO}_2 + 2e^- 
\]

Electrons liberated in this reaction are first transferred to flavodoxin and under anaerobic conditions further to metronidazole (imidazoles) thus reducing the drug to its bactericidal form.
by metronidazole. The short-lived reaction products formed can react with DNA, causing strand breaks and subsequent cell death. On the other hand, acetyl-CoA produced in the oxidation of pyruvate is mainly metabolized to a catabolic end-product, acetate. Accordingly, the measurement of acetate formation can be used to estimate the dependence of the reductive activation of metronidazole on the oxidation of pyruvate.

The metabolic functions linked to the activation of nitroimidazoles in H. pylori and factors leading to resistance in some bacterial strains are still unknown. Therefore, the aim of this study was to investigate pyruvate metabolism and acetate production in H. pylori and the dependence of the reductive activation of metronidazole on these specific metabolic activities. Purification of H. pylori flavodoxin, the electron transfer protein directly linked to the reductive activation of metronidazole, is also reported.

Materials and methods

Preparation of bacterial extracts

NCTC 11637, NCTC 11638 and nine H. pylori strains, obtained from Finnish patients by gastroscopy, were grown on brucella agar plates (BBL, Cockeysville, MD, USA) supplemented with whole horse blood (7%) in an atmosphere of 5% O2, 10% CO2 and 85% N2 at 35°C for 2 days. The bacteria recovered at endoscopy were identified as H. pylori based on colony morphology, Gram staining and positive urease, catalase and oxidase tests. The sensitivity of the strains to metronidazole was assayed by disc diffusion method and E tests. The cultured bacteria were ruptured by sonication and bacterial extracts (supernatant) were then obtained by centrifugation at 100,000g for 60 min at 5°C.

Purification of flavodoxin

Flavodoxin was purified from bacterial extracts of strain NCTC 11637 by using anion exchange20 and gel filtration chromatography. Soluble protein (35–50 mg) was passed through a Sepharose Q column (2 mL) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Flavodoxin bound to the top of the column as a yellow band. The column was first washed with 0.25 M NaCl in the same buffer and bound flavodoxin was eluted by increasing the salt concentration linearly to 0.35 M. Pooled flavodoxin fractions were diluted with an equal amount of 50 mM Tris-HCl buffer, pH 7.5, and subjected to further chromatography on Sepharose Q. After the second purification step, the concentrated flavodoxin solution was applied to a Superose 12HR gel filtration column (HPLC), which was equilibrated with 50 mM Tris-HCl buffer, pH 7.5 and eluted with the same buffer. Flavodoxin was identified and quantified by measuring its absorption spectrum.21 Its function as an electron acceptor in pyruvate oxidation by H. pylori was discerned from spectral changes (300–700 nm) measured after the incubation of the protein (100 μg) with cell extract in the presence of 10 mM pyruvate and 0.1 mM coenzyme A (CoA).

Spectrophotometric assays of pyruvate:flavodoxin oxidoreductase activity

In spectrophotometric assays oxygen was removed by bubbling oxygen-free nitrogen into reaction mixtures. A cuvette contained 50 mM Tris-HCl, pH 7.5, 50 μM TPP, 0.1 mM MgCl2, 5 mM sodium pyruvate, 1 mM methyl viologen (MV), 50 μM coenzyme A (CoA) and 100–200 μg of bacterial extract in a total volume of 1 mL at room temperature. After a short lag period the absorbance of reduced MV increased steadily, and pyruvate:flavodoxin oxidoreductase activity was calculated from the linear part of the reaction curve. The k0 of MV was taken as 13 mM-1·cm-1.22 As H. pylori is a microaerophilic bacterium and possesses cytochrome c552.15 we decided to study pyruvate:flavodoxin oxidoreductase activity aerobically from cell extracts of the strain NCTC 11637 by monitoring the reduction of cytochrome c at 25°C.23 Each cuvette contained 50 mM Tris-HCl, pH 7.5, 50 μM TPP, 50 μM bovine heart cytochrome c, 10 mM sodium pyruvate and bacterial extract. Reactions were started by adding 10 μL of 5 mM CoA to 1 mL of reaction mixture. Other α-ketoacid reductase activities were determined by using different concentrations of α-ketobutyrate and α-ketoglutarate as substrates. Pyruvate:flavodoxin oxidoreductase activity of intact bacteria was measured in the presence of pyruvate as development of a blue colour of reduced MV (10 mM) anaerobically or without removing dissolved oxygen from the incubation medium. Oxygen concentration was measured using a Clark-type oxygen electrode covered with a Teflon membrane.

A cetate production and metronidazole reduction assays

A cetate production and metronidazole reduction were assayed by incubating bacteria in 50 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 10 mM pyruvate and 0.2 mM metronidazole buffer at 37°C for 4 h. When bacterial extracts were used, the buffer also contained 0.1 mM CoA and 50 μM TPP. In anaerobic assays the incubation buffer and microbial suspensions were flushed with nitrogen before the reaction was started by adding bacteria to the reaction mixture. A cetate was analysed using a commercial test kit (acetic acid U.V method, Boehringer Mannheim, Germany). Metronidazole reduction was determined spectrophotometrically by measuring the decrease in absorbance at
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320 nm (ε = 9.31 mM⁻¹ cm⁻¹). The effect of adenosine phosphates on acetate generation was measured from bacterial extracts of strain NCTC 11637 with 2 mM AMP, ADP or ATP in the incubation mixture.

A nalytical methods

Protein was estimated by the Lowry method²⁴ with bovine serum albumin as a standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 0.5 mm thin horizontal ExelGels (Pharmacia, Sweden) with an 8–18% gradient. Protein bands were detected by colloidal Coomassie Brilliant Blue G-250 staining with sensitivity comparable to that of a silver stain.²⁵

Results

H. pylori flavodoxin was purified to apparent homogeneity from extracts of strain NCTC 11637 by three successive chromatography steps (two involving anion exchange on Superose Q and one gel filtration on Superose 12HR). From its profile of elution from the gel filtration column (Figure 1), the size of the protein was estimated as 20 kDa. SDS-PAGE analysis revealed one major band with a molecular weight of 18 kDa (results not shown). The estimated values were similar to the reported sizes of some bacterial flavodoxins.²¹ Spectrophotometric measurements revealed that the purified protein was an electron transfer protein, as it contained flavin as a prosthetic group. To confirm whether the flavoprotein acted as an electron acceptor in pyruvate oxidation, spectral changes of the oxidized protein, from 300 nm to 700 nm, were measured in the presence of pyruvate and a catalytic amount of bacterial extract. Flavodoxin was reduced to a neutral form of flavin semiquinone by the electrons generated in the oxidation of pyruvate (Figure 2). These results indicate that H. pylori had pyruvate:flavodoxin oxidoreductase activity. This enzyme activity was detected from bacterial extracts of metronidazole-sensitive and -resistant strains as a pyruvate- and CoA-dependent reduction of MV, which substituted for flavodoxin as an electron acceptor in pyruvate oxidation. In anaerobically assayed bacterial extracts of the susceptible strain NCTC 11637, pyruvate oxidoreductase had a specific activity of 18 nmol/min/mg. Two metronidazole-resistant and two metronidazole-sensitive strains showed lower pyruvate:flavodoxin oxidoreductase activities. The maximal MV reduction rates, as compared with the type strain, were 30% and 72% (resistant bacteria) and 37% and 98% (sensitive bacteria). Pyruvate:flavodoxin oxidoreductase activity was detected also in intact bacteria (NCTC 11637 and NCTC 11638) as a development of a dark blue colour of reduced MV. Even in the presence of molecular oxygen the blue colour appeared within 10–15 min in these bacterial suspensions, probably after the reduction of dissolved oxygen in completely filled and tightly sealed glass tubes.

Pyruvate:flavodoxin oxidoreductase activity in bacterial extracts was additionally detected as the reduction of cytochrome c in the presence of atmospheric oxygen. No changes in cytochrome c spectra at 550 nm were observed when either pyruvate or CoA were omitted from the reaction mixture. Flavodoxin was essential for pyruvate oxidation, catalysing electron transfer from pyruvate: flavodoxin oxidoreductase to cytochrome c. When flavodoxin was removed from the bacterial extracts of strain NCTC 11637 by anion exchange chromatography, the rate of cytochrome c reduction was only 15% of the maximal rate. The residual activity may have resulted from direct

![Figure 1. Profile of H. pylori flavodoxin elution from a Superose 12HR gel filtration column. Arrows indicate elution of molecular weight standards (44,000, 17,000 and 1350 Da).](image1)

![Figure 2. A bsorption spectra of oxidized (---) and reduced (-- -- --, semiquinone form) H. pylori flavodoxins. Flavodoxin was reduced by the electrons generated in the oxidation of pyruvate.](image2)
transfer of electrons to cytochrome c from reduced pyruvate:flavodoxin oxidoreductase. Strain NCTC 11637 also oxidized \( \alpha \)-ketoacids other than pyruvate (Table). The low apparent \( K_m \) for \( \alpha \)-ketoglutaric acid (23 \( \mu \)M) indicates that this essential metabolic intermediate is a natural substrate for \( \alpha \)-ketoacid:flavodoxin oxidoreductase in \( H. \) pylori.

The main metabolic end-product observed in pyruvate oxidation by \( H. \) pylori was acetate. Anaerobically incubated intact bacteria (10\(^9\)) of strain NCTC 11637 produced 120 ± 20 nmol of acetate/h at a saturating concentration of pyruvate (10 mM). In the presence of atmospheric oxygen \( H. \) pylori excreted twice as much acetate (240 ± 10 nmol/h). The higher acetate production presumably resulted from the accelerated enzyme activity of pyruvate:flavodoxin oxidoreductase due to the rapid reoxidation of the enzyme itself or flavodoxin by molecular oxygen. To discover more about acetate metabolism in \( H. \) pylori, the effect of adenosine phosphates on acetate formation was examined. Incubation of the bacterial extracts with 2 mM ADP increased the rate of acetate production by 23%, whereas both 2 mM AMP and 2 mM ATP decreased acetate formation by approximately one-third as compared with controls. These results suggest that the reaction product of pyruvate: flavodoxin oxidoreductase activity, acetyl-CoA, is probably further metabolized to acetate by an ADP-dependent acetyl-CoA synthetase or by the combined action of phosphotransacetylase and acetate kinase. The cause of the decrease in acetate production was probably competitive inhibition by AMP and product inhibition by ATP.

When metronidazole-sensitive and -resistant strains were incubated anaerobically in the presence of pyruvate and metronidazole, all bacteria reduced the drug (18 ± 2.0 nmol/h, 10\(^9\) bacteria). A significant positive correlation between acetate production and metronidazole reduction was observed (\( r = 0.77, P < 0.01, n = 11; \) see Figure 3). Reduction of metronidazole was dependent on the amount of substrate, drug and bacteria when assayed for strain NCTC 11637. Metronidazole reduction and acetate production decreased when pyruvate concentrations were <5 mM. In the presence of atmospheric oxygen, metronidazole was not activated although acetate production was twice as high as in anaerobic conditions.

**Discussion**

Understanding of pyruvate metabolism in \( H. \) pylori may lead to the development of more effective strategies for eradicating this pathogen. NMR spectroscopy studies have revealed that \( H. \) pylori produces acetate, lactate, ethanol, alanine, formate and CO\(_2\) from pyruvate. In this study, acetate, small amounts of ethanol and lactate have been detected in broth cultures of strain NCTC 11637. Recent studies confirm an earlier observation that pyruvate is oxidized mainly by CoA-dependent pyruvate: flavodoxin oxidoreductase. The reaction product, acetyl-CoA, is metabolized further to acetate by acetyl-CoA synthetase or by the combined action of phosphotransacetylase and acetate kinase. However, the conversion of pyruvate to acetate and formate suggests that a mixed-acid fermentation pathway also exists. Ethanol is produced by NADP-dependent alcohol dehydrogenase, which has been purified and characterized from the type strain NCTC 11637. After removal of flavodoxin from bacterial extracts, a soluble, NAD-dependent lactate dehydrogenase has been observed. In the presence of an excess nitrogen source, urea or glutamate, \( H. \) pylori converts pyruvate to alanine by the action of alanine transaminase. These reactions are presented schematically in Figure 4.

For nitroimidazoles to be bactericidal, their nitro group has to be reduced. The oxidation of pyruvate, as presented above, suggests that in \( H. \) pylori this reduction occurs via the pyruvate:flavodoxin oxidoreductase complex. In this reaction chain the nitroimidazole acts as an electron sink by capturing electrons from reduced flavodoxin (Figure 4). This view is supported by the finding that bacterial extracts depleted of flavodoxin lose their ability to reduce metronidazole.

The production of formate from \( H. \) pylori has been reported. Although the metabolic route from pyruvate to formate is not known in detail, it is possible that pyruvate: flavodoxin oxidoreductase is replaced by pyruvate-formate lyase in metronidazole-resistant strains. In this case, electron flow to flavodoxin and CO\(_2\) generation would be replaced by the release of formate, and pyruvate oxidation would be detached from flavodoxin and concomitant metronidazole reduction. A nither pathway that avoids the activation of imidazoles is the reduction of

**Table. Flavodoxin dependent reduction of cytochrome c by various \( \alpha \)-ketoacids**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent ( K_m ) (( \mu )M)</th>
<th>Maximal relative reaction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate (( \alpha )-ketopropionic acid)</td>
<td>310</td>
<td>100</td>
</tr>
<tr>
<td>( \alpha )-K etobutyric acid</td>
<td>4000</td>
<td>5</td>
</tr>
<tr>
<td>( \alpha )-K etoglutaric acid</td>
<td>23</td>
<td>31</td>
</tr>
</tbody>
</table>

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pyruvate to lactate instead of its metabolism to acetyl-CoA. High lactate dehydrogenase activity, compensating for decreased pyruvate:flavodoxin oxidoreductase activity, may result in metronidazole:flavodoxin oxidoreductase activity, which can be observed even in the metronidazole-resistant strain NCTC 11637. A similar mechanism may exist in *H. pylori*, since weak lactate dehydrogenase activity has been detected even in the metronidazole-sensitive type strain NCTC 11638, and from nine *H. pylori* strains isolated from Finnish patients.

It is important to clarify whether metronidazole susceptibility and resistance in *H. pylori* are linked to differing expression of pyruvate metabolizing enzymes. The most commonly used regimen in the treatment of *H. pylori* infection has been triple therapy, including bismuth compounds and imidazoles (metronidazole). It has been reported that bismuth inhibits respiratory chain phosphorylation and reduces intracellular ATP levels in *H. pylori*. In our study, added ADP activated and ATP reduced acetate production from pyruvate, probably by stimulating and suppressing acetyl-CoA synthetase or phosphotransacetylase/acetate kinase activities. Therefore, the cooperative action of bismuth compounds and metronidazole may result from the decreased ATP generation via respiratory chain phosphorylation, and from the compensating and accelerated substrate level ATP production. CoA, a necessary cofactor in the oxidation of pyruvate by pyruvate:flavodoxin oxidoreductase, is recycled more rapidly, and this could lead to an accelerated activity of pyruvate:flavodoxin oxidoreductase and simultaneously to a higher reduction rate of flavodoxin, and ultimately to a more efficient activation of imidazoles.

*H. pylori* activates imidazoles only under anaerobic conditions: no reduction is observed in the presence of atmospheric oxygen. This suggests that oxygen—the best biological electron acceptor known—prevents the activation of metronidazole by capturing electrons directly from reduced flavodoxin or pyruvate:flavodoxin oxidoreductase. Oxygen may also rapidly remove the electron from the activated imidazole reforming the original, non-toxic drug (futile cycle). It has been suggested that lowered capacity for oxygen consumption and a higher intracellular oxygen concentration in the metronidazole-resistant strain of *Trichomonas vaginalis*, permits more effective futile cycling between the cytotoxic product of metronidazole and molecular oxygen. If intracellular oxygen were a major cause of imidazole resistance in *H. pylori*, it might be possible to enhance metronidazole reduction in *H. pylori* by combining the drug with oxygen scavengers such as vitamin C. This could make conditions

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**Figure 3.** Correlation between acetate production from pyruvate and concomitant metronidazole reduction by *H. pylori* under anaerobic conditions (*r* = 0.77, *P* < 0.01, *n* = 11; 10⁵ bacteria). The reductive activation of metronidazole was assayed from type strains NCTC 11637 and NCTC 11638, and from nine *H. pylori* strains isolated from Finnish patients.

**Figure 4.** Schematic view of pyruvate metabolism and the reductive activation of metronidazole by *H. pylori*. Under anaerobic conditions electrons (e⁻) liberated in the oxidation of pyruvate by pyruvate:flavodoxin oxidoreductase are transferred via flavodoxin to metronidazole (5-nitroimidazoles, - - - - -) leading to the activation of the drug.
in the cytoplasm sufficiently reducing for more effective activation of metronidazole under the microaerophilic conditions prevailing in the stomach.

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