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Identification of Carboxylation Enzymes and Characterization of a Novel Four-Subunit Pyruvate:Flavodoxin Oxidoreductase from *Helicobacter pylori*

NICKY J. HUGHES,[1,2] PETER A. CHALK,[2] CHRIS L. CLAYTON,[2] AND DAVID J. KELLY[1]*

Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH,[1] and Glaxo Research and Development Ltd., Stevenage, Herts SG1 2NY,[2] United Kingdom

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The enzyme activities responsible for carboxylation reactions in cell extracts of the gastric pathogen *Helicobacter pylori* have been studied by $H^{14}CO_3^-$ fixation and spectrophotometric assays. Acetyl coenzyme A carboxylase (EC 6.4.1.2) and malic enzyme (EC 1.1.1.40) activities were detected, whereas pyruvate carboxylase (EC 6.4.1.1), phospho*enol*pyruvate carboxylase (EC 4.1.3.1) and phospho*enol*pyruvate carboxykinase (EC 4.1.1.49) activities were absent. However, a pyruvate-dependent, ATP-independent, and avidin-insensitive $H^{14}CO_3^-$ fixation activity, which was shown to be due to the isotope exchange reaction of pyruvate:flavodoxin oxidoreductase (EC 1.2.7.1), was present. The purified enzyme is composed of four subunits of 47, 36, 24, and 14 kDa. N-terminal sequence analysis showed that this enzyme is related to a recently recognized group of four-subunit pyruvate:ferredoxin oxidoreductases previously known only from hyperthermophiles. This enzyme from *H. pylori* was found to mediate the reduction of a number of artificial electron acceptors in addition to a flavodoxin isolated from *H. pylori* extracts, which is likely to be the in vivo electron acceptor. Indirect evidence that the enzyme is capable of in vitro reduction of the anti-*H. pylori* drug metronidazole was also obtained.

Helicobacter pylori is a microaerophilic, gram-negative, motile, curved or spiral bacterium, isolated from the mucous layer overlying the human gastric epithelium (35, 53). To date, eleven *Helicobacter* species have been isolated from a range of mammals (15, 20, 32). The organism is now accepted as the etiological agent for type B gastritis, and extensive evidence suggests a link with duodenal and gastric ulcer diseases (17, 21, 25, 47, 52). Individuals with long-term infections may also be at increased risk of developing gastric carcinoma (44).

Enzyme activities identified in this bacterium include catalase, cytochrome oxidase, superoxide dismutase, and phospholipase, and it is a constitutive producer of a potent urease (18, 23). Pathogenicity has been associated with adhesion to the gastric epithelium (7, 24, 43), urease production (36), a vacuolating cytotoxin (10, 11, 13), and the action of phospholipases (31). However, knowledge of the basic metabolism of *H. pylori* is meager. This organism exhibits limited glucose utilization (39, 41), but it is reported to contain pentose phosphate (38) and Entner-Douderoff pathways (8, 42). Recently, utilization of glucose as a carbon and energy source in a defined medium has been shown (48). Active fumarate catabolism, generating malate and succinate from fumarate initially, followed by the formation of lactate, acetate, formate, and alanine, has been demonstrated (40). Suspensions of intact cells have been shown to generate lactate, ethanol, alanine, acetate, and CO_2 from pyruvate (8). This organism also requires elevated levels of CO_2 for growth, and it has been suggested that potent urease activity in part feeds this requirement (22). HCO_3^- is used in de novo synthesis of pyrimidines, but the demand for CO_2 considerably exceeds that of HCO_3^-. Gaseous CO_2 cannot be replaced by the addition of bicarbonate to the medium (22).

Microorganisms exhibiting dependence on elevated CO_2 levels are termed capneic or capnophilic. Perhaps the best examples of pathogenic capnophiles are *Neisseria* spp. The enzymes known to be involved in CO_2 assimilation by *Neisseria* spp. are carbonic anhydrase (34) and phospho*enol*pyruvate (PEP) carboxylase (12). Pyruvate carboxylase, PEP carboxykinase, and PEP carboxytransphosphorylase have not been detected (26). Kinetic data for PEP carboxylase in *Neisseria gonorrhoeae* indicate that carboxylation can occur at very low concentrations of bicarbonate, and it is considered unlikely that this enzyme is the cause of CO_2 dependence. In *Capnocytophaga* species, increased CO_2 is thought to be necessary because these bacteria depend on PEP carboxykinase to synthesize ATP and oxaloacetate. There is therefore net incorporation of CO_2 during catabolism in these organisms (27).

Little is known about the requirement for and metabolic fate of CO_2 in *H. pylori*. Therefore, we were prompted to investigate the primary fixation reactions of CO_2 (and HCO_3^-) to elucidate the reasons for the fastidious gaseous requirements of this bacterium, with the ultimate goal of discovering improved anti-*H. pylori* agents. In this work, we demonstrate the presence in *H. pylori* of pyruvate:flavodoxin oxidoreductase (POR) activity by virtue of its catalysis of an isotope exchange reaction between $H^{14}CO_3^-$ and pyruvate. Pyruvate:acceptor oxidoreductases carry out thiamine PP_i-dependent oxidative decarboxylation of pyruvate to form acetyl coenzyme A (acetyl-CoA) and are oxygen sensitive and most commonly found in obligate anaerobes, for example, *Clostridium acetobutylicum* (37) and *Trichomonas vaginalis* (54). They are commonly single-subunit enzymes, although the enzyme purified

* Corresponding author. Mailing address: Department of Molecular Biology and Biotechnology, University of Sheffield, P.O. Box 594, Firth Court, Western Bank, Sheffield S10 2UH, United Kingdom. Phone: 44 114 282 4414. Fax: 44 114 272 8697.

from the aerobic archaeon *Halobacterium halobium* is a heterodimer (28), and the corresponding genes have been cloned and sequenced (45). The purification and some properties of *H. pylori* POR, including indirect evidence of this enzyme's ability to effect reduction of the drug metronidazole, which is used in the treatment of *H. pylori* infections, are reported here. Remarkably, this enzyme is shown to be related to a newly recognized group of four-subunit PORs thus far known only from some hyperthermophilic bacteria and archea.

MATERIALS AND METHODS

Enzymes and chemicals. $NaH^{14}CO_3$ (1.85 GBq/mmol) was purchased from Amersham International PLC. Purified *Rhodobacter capsulatus* pyruvate carboxylase was obtained from D. J. Kelly, University of Sheffield. Purified *Clostridium pasteurianum* ferredoxin was purchased from Sigma. Vancomycin (Vancocin) was obtained from Eli Lilly, and amphotericin B (Fungizone) was obtained from Squibb. Molecular mass markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Amersham, and precast gels were purchased from Bio-Rad. All other enzymes and chemicals were purchased from Sigma.

Bacterial strains, growth conditions, and cell extract preparation. *H. pylori* NCTC 11637 was obtained from the National Collection of Type Cultures, Colindale, United Kingdom, and maintained at 37°C on Columbia agar supplemented with 5% chocolate horse blood and 10 μg each of vancomycin, amphotericin B, and polymyxin B ml^{-1} in a microaerophilic atmosphere generated in gas jars by Campypaks (BBL). For small-scale enzyme studies, bacteria were grown overnight with agitation in 25 ml of brain heart infusion broth supplemented with 5% (vol/vol) fetal calf serum and the antibiotics mentioned immediately above in 100-ml acid-washed flasks contained within microaerophilic gas jars. Cells were harvested by centrifugation, and the pellet was resuspended in 20 mM Tris-HCl (pH 8.0) and sonicated with five bursts of 15 s at 10 A (Soniprep 150). After centrifugation at 14,000 × *g* and 4°C for 10 min, the supernatant (cell extract) was dialyzed in 20 mM Tris-HCl (pH 8.0) buffer and kept on ice for use in enzyme assays on the same day.

$H^{14}CO_3^-$ fixation assays. Incorporation of $NaH^{14}CO_3$ into acid-stable products was monitored in the presence of various substrates and cofactors. In all assays, *H. pylori* cell extract (2 mg of protein) was incubated at 37°C in a reaction mixture (250 μl, final volume) containing 1 mM (0.5 μCi) $NaH^{14}CO_3$, 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and the relevant substrates and coupling enzymes listed below in glass test tubes (1 by 10 cm) in a heating block. After 1 h, the mixture was acidified by the addition of 50 μl of 12 M formic acid and heated at 80°C for 45 min. An aliquot (200 μl) was then added to 5 ml of a scintillant (Safefluor S; Lumac, Groningen, Netherlands) and counted for 5 min on an LKB1219 Rackbeta liquid scintillation counter. The substrate additions were as follows: for pyruvate carboxylase, 10 mM Na pyruvate, 1 mM ATP, 0.1 mM acetyl-CoA, 5 U of malate dehydrogenase (Sigma), and 1 mM NADH; for PEP carboxylase, 10 mM PEP, 1 mM NADH, and 5 U of malate dehydrogenase; for PEP carboxykinase, the same as for PEP carboxylase with the addition of 1 mM ADP; for malic enzymes, 10 mM KCl, 10 mM D-(+)- or L-(−)-malate, and 1 mM NAD or NADP; for acetyl-CoA carboxylase, 0.2 mM acetyl-CoA, 10 mM KCl, and 1 mM ATP.

Spectrophotometric carboxylase enzyme assays. Pyruvate carboxylase, PEP carboxylase, and PEP carboxykinase activities were monitored by assays linked to NADH-dependent reduction of oxaloacetate by malate dehydrogenase. For pyruvate carboxylase, each 1-ml assay contained 100 mM Tris-HCl (pH 8.0), 10 mM Na pyruvate, 3.3 mM Na-ATP, 20 mM $NaHCO_3$, 6.6 mM $MgCl_2$, 0.15 mM NADH, 10 U of malate dehydrogenase, and cell extract. The reaction was initiated by the addition of pyruvate and ATP, and the disappearance of NADH was monitored continuously at 340 nm. PEP carboxylase and PEP carboxykinase activities were measured by 1-ml assays containing 100 mM Tris-HCl (pH 7.4), 75 mM $NaHCO_3$, 10 mM PEP, 10 mM $MgCl_2$, 5 mM ADP, 0.3 mM NADH, 10 U of malate dehydrogenase, and cell extract. Purified *R. capsulatus* pyruvate carboxylase and corn PEP carboxylase were used as positive controls.

POR assays. For rapid identification of POR-containing fractions under essentially aerobic conditions for purification purposes, 50 to 100 μl of each fraction was added to the wells of a microtiter plate which also contained 8 μl each of 500 mM Na pyruvate, 10 mM CoA, and 100 mM methyl viologen (MV). POR-containing fractions rapidly turned blue, i.e., before reduced MV was eventually reoxidized by atmospheric oxygen. For quantitative measurement of rates of MV reduction, a modified version of the assay described by Blamey and Adams (3) was employed. The standard 2-ml assay contained 0.1 mM CoA, 1 mM MV, 5 mM Na pyruvate, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.0). Reactions were carried out in sealed 3-ml cuvettes gassed with oxygen-free nitrogen. The reduction of MV at 600 nm was measured with a Lambda 5 spectrophotometer (Perkin-Elmer) at 30°C. Results were expressed in units mg of $protein^{-1}$, where one unit of activity equals the reduction of 1 μmol of MV min^{-1}. Protein concentrations were determined by using Coomassie protein determination reagent (Pierce), with bovine serum albumin as the standard. The pH optimum for POR activity was measured by the standard assay in 50 mM morpholineethanesulfonic acid-Tris buffer, between pH values of 5.0 and 10.0. The temperature optimum was measured by preequilibrating the enzyme assay buffer at the required temperature for 10 min.

Purification and N-terminal sequencing of POR. For large-scale POR purification, 14 g (wet weight) of cells previously harvested and stored at −70°C was resuspended in 10 ml of buffer A (50 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol). Cells were broken by sonication (5 cycles of 30 s on and 60 s off at 12 A). All buffers used during purification were flushed with N_2, filtered, and then degassed. Cell membranes were removed by centrifugation at 4°C for 1 h at 100,000 × *g*, and the supernatant was loaded onto a Mono Q 10/10 column (Pharmacia) previously equilibrated in buffer A. The column was washed with 5 column volumes of buffer A, and POR activity was eluted with a 0 to 300 mM gradient of NaCl in buffer A. Then an aliquot of each fraction was assayed for POR. Pooled peak fractions were diluted with an equal volume of buffer A and loaded onto a Green A dye affinity column (1 by 12.7 cm; Amicon) previously equilibrated with buffer A. The column was eluted with a linear gradient from 0 to 1 M NaCl over 9 column volumes. Ammonium sulfate was added to pooled active fractions to a concentration of 1.5 M, filtered through a 0.2-μm-pore-size membrane, and loaded onto a phenyl Superose 5/5 column (Pharmacia) equilibrated in buffer B (buffer A containing 1.5 M ammonium sulfate). The column was developed with a decreasing linear ammonium sulfate gradient from 1.5 to 0 M. Active fractions were concentrated on a Centriprep 10 filter (Amicon) to 0.2 ml and loaded onto a Superose 12 column (Pharmacia) equilibrated in buffer A. Active fractions were examined by SDS-PAGE. For N-terminal sequencing, fractions were separated on 4 to 15% polyacrylamide gradient gels and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was stained with amido black, and individual bands were excised for N-terminal sequencing by the automated Edman degradation procedure.

Flavodoxin isolation. Cells were broken and cell extracts were prepared as described above for purification of POR, except that buffer A was replaced with 50 mM Tris-HCl (pH 8.0). The sample was loaded onto a Mono Q 10/10 column (Pharmacia) preequilibrated in the same buffer, washed with 5 column volumes of buffer, and eluted with a linear gradient of NaCl from 0 to 1 M. Absorption spectra of visibly colored fractions were obtained over a wavelength range of 200 to 600 nm.

TABLE 1. $H^{14}CO_3^-$ fixation assay results for acetyl-CoA carboxylase

Assay system	Incorporation from $H^{14}CO_3^-$ after 60-min incubation (dpm)[a]
Complete system	2,246
Minus cell extract	38
Minus acetyl-CoA	40
Minus ATP	19
Minus $MgCl_2$	54
Minus KCl	2,122
Plus avidin[b]	62
Plus avidin and biotin[c]	2,049

[a] Data are means of two assays, containing 2 mg of cell extract, for incorporation of $H^{14}CO_3^-$ into acid-stable products in the presence of substrates and cofactors of acetyl-CoA carboxylase.
[b] Cell extract was preincubated with 1 U of avidin (30 min, 37°C).
[c] One unit of avidin was incubated with excess biotin (10 min, 37°C) prior to incubation with cell extract (30 min, 37°C).

RESULTS

Fixation of $H^{14}CO_3^-$ by cell extracts. The results of a series of experiments to measure substrate-dependent fixation of $H^{14}CO_3^-$ into acid-stable products catalyzed by cell extracts are shown in Tables 1 through 3. Acetyl-CoA carboxylase activity which was dependent on the presence of all of the substrates normally essential for this reaction, except for KCl, was detected (Table 1). This activity was abolished by preincubating the enzyme with 1 U of avidin, but inhibition was relieved when the extract was preincubated with excess biotin before the addition of avidin (Table 1). NADP-dependent, L-(−)-malic enzyme activity was also present (Table 2). This activity displayed some dependence on $MgCl_2$ but did not require KCl for full activity. No PEP-dependent $H^{14}CO_3^-$ fixation due to PEP carboxylase or PEP carboxykinase was demonstrated (data not shown), and no activity was detected when cells were

TABLE 2. $H^{14}CO_3^-$ fixation assay results for malic enzymes

Assay system	Incorporation from $H^{14}CO_3^-$ after 60-min incubation (dpm)[a]			
	L-(−)-Malate–NADP	L-(−)-Malate–NAD	D-(+)-Malate–NADP	D-(+)-Malate–NAD
Complete system	1,235	457	526	575
Minus cell extract	489	531	562	595
Minus malate	528	520	558	566
Minus NADP-NAD	534	517	611	509
Minus $MgCl_2$	793	541	571	576
Minus KCl	1,121	595	663	614

[a] Data are means of two assays examining incorporation of $H^{14}CO_3^-$ into acid-stable products in the presence of substrates and cofactors for malic enzymes. Two milligrams of cell extract protein was used for each assay. Both D and L isomers of malate were tested with NAD and NADP.

resuspended in MOPS (morpholinepropanesulfonic acid) buffer, instead of Tris-HCl.

The highest $NaH^{14}CO_3$ incorporation by cell extracts was seen in the assay designed to detect pyruvate carboxylase (Table 3). However, the apparent carboxylation activity required only pyruvate and either acetyl-CoA or CoA and was independent of ATP. There was a 50% decrease in incorporation when $MgCl_2$ was omitted from the system, but this activity was not affected by omission of malate dehydrogenase or NADH (included to shift the equilibrium of the pyruvate carboxylase reaction by the removal of oxaloacetate). Finally, this activity was not inhibited by the addition of avidin, indicating that this activity is not biotin linked and therefore cannot in fact be due to pyruvate carboxylase. The results are, however, consistent with an isotope exchange reaction between the carboxyl group of pyruvate and radiolabelled bicarbonate, which is known to be catalyzed by pyruvate:acceptor oxidoreductases (46). The presence of such an enzyme in *H. pylori* was subsequently confirmed by the ability of cell extracts to catalyze pyruvate- and CoA-dependent reduction of MV, with a specific activity of 0.030 units mg of protein^{-1}. In the absence of either pyruvate or CoA, no MV reduction was observed. When pyruvate was replaced with 2-oxoglutarate, MV reduction was also observed, but with a lower specific activity (0.015 units mg of protein^{-1}).

Fractions from ion-exchange chromatography on Mono Q columns were assayed for both pyruvate-dependent $H^{14}CO_3^-$ isotope exchange and MV reduction. Both activities were coeluted at 0.2 M NaCl (Fig. 1). No biotin-containing proteins were detected when these fractions were run on SDS-PAGE gels, blotted, and probed with avidin-horseradish peroxidase (Fig. 2 and data not shown).

Spectrophotometric assays for carboxylation enzymes. Spectrophotometric assays for PEP carboxylase, PEP carboxykinase, and pyruvate carboxylase detected no activity in *H. pylori* cell extracts, confirming $H^{14}CO_3^-$ fixation results.

Detection of biotinylated polypeptides in cell extracts. Several carboxylases, most notably, pyruvate carboxylase and acetyl-CoA carboxylase, contain covalently bound biotin at their active sites. Western blots (immunoblots) of *H. pylori* cytoplasmic proteins probed with avidin-horseradish peroxidase revealed three bands (Fig. 2). The most abundant biotinylated protein, which is likely to be the biotin-containing subunit of acetyl-CoA carboxylase, had a molecular mass of 24 kDa. Other minor bands were detected at 23 and 40 kDa.

Characterization and purification of POR. By MV reduction assay, *H. pylori* POR activity in cell extracts was found to have an optimum temperature for activity of 30°C and an optimum pH of 8.0. In addition to MV, benzyl viologen was capable of coupling with this enzyme, but NAD and *C. pasteurianum* ferredoxin were unable to do so (data not shown). In some anaerobes, it is known that POR can effect the reduction of the drug metronidazole to an active form which causes cellular damage (19). Metronidazole has also been effective in the treatment of *H. pylori* infections. The effects of adding increasing concentrations of metronidazole to the MV-linked POR assay are shown in Fig. 3. Addition of this drug resulted in a lag before MV began to be reduced at a slow rate.

A purification scheme for POR activity was devised (Table 4); it yielded a final fraction that contained four protein bands (A through D) on silver-stained SDS-PAGE gels (Fig. 4). The total activity recovered following Mono Q chromatography appears to be greater than that in the original sample. This was due to the stimulation of POR activity by NaCl included in the elution buffer. Low recovery of activity was most likely due to inactivation by oxygen, to which this enzyme is inevitably exposed, despite gassing sealed tubes with nitrogen and including dithiothreitol in all buffers. Inactivation may also be due to an irreversible loss of thiamine PP_i from the enzyme during purification. However, the addition of thiamine PP_i to purification buffers resulted in no increase in enzyme stability. Storage of purified POR under liquid nitrogen for even short periods resulted in a complete loss of activity. Cell extracts were also found to be highly unstable, losing approximately 70% of POR activity after 2 weeks of storage in liquid nitrogen. N-terminal sequences of all four protein bands from an SDS-PAGE gel of the final step in purification were obtained and compared with other POR sequences (Table 5). Stretches of conserved resi-

TABLE 3. $H^{14}CO_3^-$ fixation assay results for pyruvate carboxylase

Assay system	Incorporation from $H^{14}CO_3^-$ after 60-min incubation (dpm)[a]
Complete system	31,711
Minus cell extract	81
Minus pyruvate	281
Minus ATP	29,483
Minus acetyl-CoA	4,720
Plus CoA[b]	29,148
Minus $MgCl_2$	15,774
Minus MDH[c]	31,004
Minus NADH[c]	30,791
Plus avidin[d]	31,286
Plus biotin and avidin[e]	29,702

[a] Data are means of two assays examining incorporation of $H^{14}CO_3^-$ into acid-stable products in the presence of substrates for pyruvate carboxylase. Two milligrams of cell extract protein was used for each assay.

[b] CoA replaced acetyl-CoA.

[c] Malate dehydrogenase (MDH) and NADH were included to shift the equilibrium by the removal of oxaloacetate.

[d] Cell extract was preincubated with 2.5 U of avidin (30 min, 37°C).

[e] Avidin (2.5 U) was incubated with excess biotin (10 min, 37°C) prior to incubation with cell extract (30 min, 37°C).

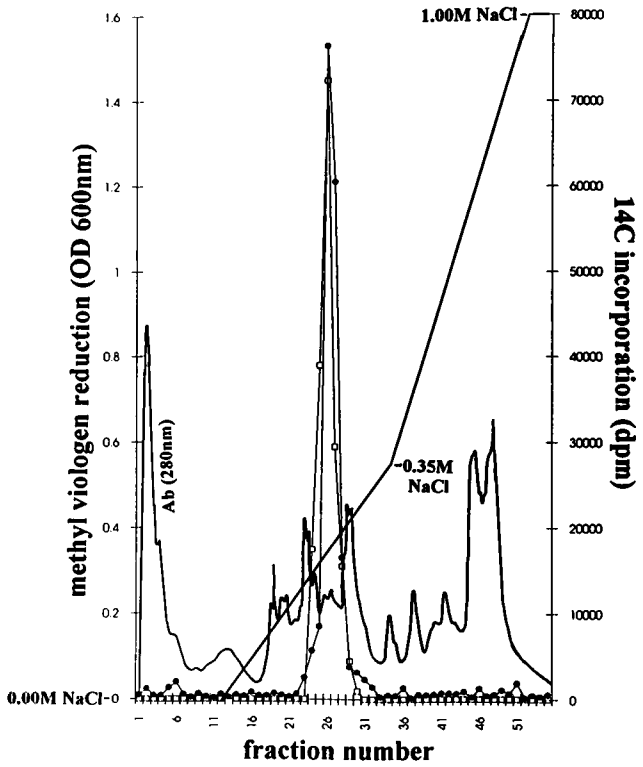

FIG. 1. Coelution of pyruvate-dependent $H^{14}CO_3^-$ fixation activity and pyruvate-dependent MV reduction from Mono Q HR 5/5. Cell extract (20 mg of protein) was partially purified on a Mono Q HR 5/5 ion-exchange column as described in Materials and Methods. Protein was eluted by an increasing salt gradient between 0 and 1 M NaCl, and elution was monitored at 280 nm (solid lines). Fractions were assayed for pyruvate- and CoA-dependent $H^{14}CO_3^-$ fixation (●) and pyruvate-dependent MV reduction (□) by microtiter plate assays. OD, optical density.

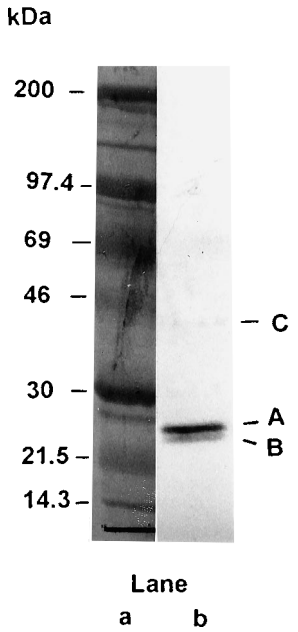

FIG. 2. Detection of biotinylated polypeptides in cell extracts of *H. pylori*. Cell extract (10 μg of protein) was loaded onto a 4 to 15% polyacrylamide gradient SDS-PAGE gel and blotted onto a PVDF membrane. The membrane was incubated with blot buffer (150 mM NaCl, 1 mM $CaCl_2$, 50 mM Tris-HCl [pH 7.6]) supplemented with 3% (wt/vol) bovine serum albumin for 60 min at room temperature. The filter was probed for 60 min at room temperature with 30 μg of horseradish peroxidase-linked avidin per ml in blot buffer and subsequently washed five times with blot buffer. The blot was developed with chloronaphthol as the substrate. Lane a, molecular mass markers and amido black stain of the PVDF membrane. Lane b, biotinylated proteins (band A, 24 kDa; band B, 23 kDa; and band C, 40 kDa).

dues previously observed in four-subunit pyruvate:ferredoxin oxidoreductases from the hyperthermophilic archaebacteria *Archaeoglobus fulgidus* (30) and *Pyrococcus furiosus* (3, 4) and the hyperthermophilic bacterium *Thermotoga maritima* (4) were also conserved in *H. pylori* POR. The 47-kDa subunit also displayed sequence similarity to the single-subunit PORs of *Klebsiella pneumoniae* (6), *Anabaena* spp. (49), and *Rhodospirillum rubrum* (5). The 36-kDa protein contained a conserved cysteine arrangement found in the two-subunit *Halobacterium halobium* POR (45).

POR activity eluted from the Superose 12 gel filtration column in a single peak, and the molecular mass of the native enzyme was calculated from the elution volume to be approximately 240 kDa. This is twice the native molecular mass calculated for other four-subunit PORs but is very similar to the values for the *K. pneumoniae* and *Anabaena* (2) enzymes. No reduction of MV occurred when pyruvate was replaced with 2-oxoglutarate, indicating that this activity is catalyzed by a separate enzyme.

Reduction of an endogenous flavodoxin by pyruvate:acceptor oxidoreductase. The in vivo electron acceptor for POR is likely to be a low-redox-potential ferredoxin or flavodoxin. Visibly pigmented Mono Q fractions were assayed for the presence of flavodoxin and ferredoxin by determining their absorption spectra. The absorption spectrum of a fraction eluted at 350 mM NaCl is shown in Fig. 5 and is characteristic of flavodoxin, exhibiting peaks at 375 and 460 nm. Both of these

TABLE 4. Purification scheme of *H. pylori* POR[a]

Step	Activity (units)	Protein (mg)	Sp act (units/mg)	Recovery (%)	Purification (fold)
Cell extract	4.825	161.00	0.030	100	1
Mono Q	6.968	22.36	0.312	144	10
Green A	3.711	4.50	0.825	77	28
Phenyl Superose	0.767	0.35	2.191	16	73
Superose 6	0.242	0.10	2.415	5	81

[a] Data are for 14 g (wet weight) of cells. Units of activity are micromoles of MV reduced min^{-1}. The ε_{600} of MV was taken as 13,000 M^{-1} cm^{-1} (51).

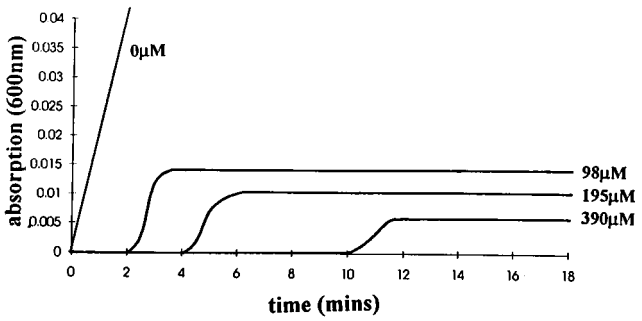

FIG. 3. Effects of metronidazole on the reduction of MV by *H. pylori* POR. Various concentrations of metronidazole were added to the standard MV-linked POR assay, as described in Materials and Methods, which contained 100 μg of cell extract. Increasing metronidazole concentrations resulted in an increased lag prior to the onset of MV reduction.

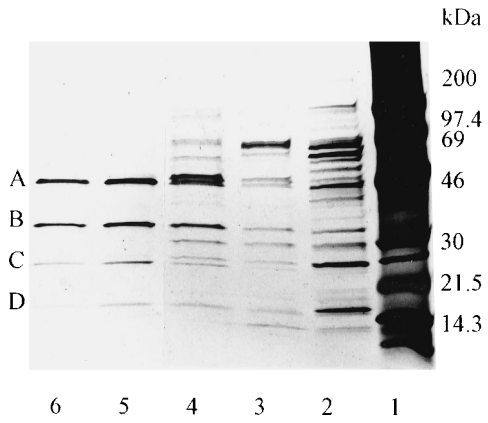

FIG. 4. SDS-PAGE analysis of fractions from *H. pylori* POR purification. One microgram of protein from each stage of purification was loaded onto a 4 to 15% SDS-PAGE gel. Protein was detected by silver staining. Lane 1, molecular mass markers; lane 2, cell extract; lane 3, Mono Q active fraction; lane 4, Green A active fraction; lane 5, phenyl Superose active fraction; lane 6, Superose 12-purified POR. Purified POR is a heterotetrameric protein composed of 47- (A), 36- (B), 24- (C), and 14-kDa (D) polypeptides.

peaks were quenched by the addition of partially purified *H. pylori* POR (active Mono Q fraction), pyruvate, and CoA (Fig. 5), and reduction was dependent on the presence of all of these components. No pigmented Mono Q fractions exhibited absorption spectra characteristic of ferredoxins.

DISCUSSION

Many pathogens are adapted to the relatively high levels of partial CO_2 pressure found in mammalian bodies. Gastric exudates have been reported to contain bicarbonate concentrations of 10 mM in dogs and 25 mM in humans (16). The mean of these two values gives a partial CO_2 pressure in gastric exudates of 71.8 mm Hg (1 mm Hg = 133.322 Pa), which corresponds to a potential concentration of aqueous CO_2 of 9.45% in the niche occupied by *H. pylori*. The very high level of urease production by this bacterium may substantially increase this concentration. Elevated CO_2 levels are vital for the growth of *H. pylori*, and this dependency does not result simply from a buffering effect of dissolved CO_2 (7a). The enzyme data reported here indicate an apparent scarcity of enzymes responsible for anaplerotic CO_2 fixation. Pyruvate carboxylase, PEP carboxylase, and PEP carboxykinase were not detected by either spectrophotometric or $H^{14}CO_3^-$ fixation assay. The explanation for the CO_2 dependence exhibited by *Capnocytophaga* spp. thus does not seem to apply to *H. pylori*. A search for the biotinylated subunits of potential biotin-linked carboxylases showed that the major biotin-containing protein in *H. pylori* has a molecular mass of 24 kDa. This is most likely the biotin carboxylase subunit of acetyl-CoA carboxylase, which catalyzes the first committed step of fatty acid biosynthesis and is thus found in many organisms (1). The presence of this enzyme in cell extracts was demonstrated by $NaH^{14}CO_3$ fixation assay, and clear inhibition by avidin confirms the biotin-dependent nature of this enzyme. Other minor biotin-containing proteins were also detected on Western blots. The 23-kDa band may be the small subunit of the propionyl-CoA carboxylase, but the size of the very weak 40-kDa band is different from those of other known biotin carboxylases, e.g., transcarboxylase contains a biotin-containing subunit of 12 kDa, while most pyruvate carboxylases have subunit molecular masses of around 120 kDa (33) or, more uncommonly, 65 kDa, as in *Pseudomonas citronellolis* (9). The existence of a malic enzyme in *H. pylori* was predicted by Mendz and Hazell (40) from the pattern of product formation following fumarate metabolism. We have confirmed the existence of such an enzyme; it appears to require L-(−)-malate and NADP as substrates.

The highest apparent $NaH^{14}CO_3$ fixation was seen in the presence of pyruvate and CoA. Since this did not require ATP and was avidin insensitive, it could not have been due to pyruvate carboxylase activity. We conclude that this apparent fixation is due to an exchange reaction between the carboxyl group of pyruvate and CO_2 catalyzed by pyruvate:acceptor oxidoreductase activity. This was confirmed by coelution of both the putative isotope exchange activity and pyruvate-dependent MV reduction activity from an ion-exchange column. This enzyme, purified 81-fold from wild-type cells by MV-linked assay, was specific for pyruvate. Thus, the 2-oxoglutarate-dependent MV reduction observed in cell extracts must be due to a separate oxidoreductase. This latter activity possibly plays a key role in the tricarboxylic acid cycle of *H. pylori*, which apparently lacks an NAD- or NADP-linked 2-oxoglutarate dehydrogenase (13a). The final preparation contained four polypeptides with molecular masses of 47, 36, 24, and 14 kDa. The *H. pylori* POR subunit composition is therefore very similar to that of *A. fulgidus* (45-, 33-, 25-, and 13-kDa subunits) (30), *P. furiosus* (45-, 31-, 24-, and 13-kDa subunits) and *T. maritima* (43-, 34-, 23-, and 13-kDa subunits) (4), and a relationship to these enzymes was confirmed by N-terminal sequence analysis. Recently, Kunow et al. (30) identified conserved residues in the N-terminal sequences of all pyruvate oxidoreductases available, regardless of subunit composition. Our results further confirm conservation among PORs and are the first demonstration of a four-subunit POR in a mesophilic eubacterium. It has been proposed that large single-subunit polypeptide pyruvate oxidoreductases have evolved by fusion from four-subunit enzymes (3). Therefore, it is perhaps surprising that *H. pylori* POR belongs to the apparently most ancestral form of this enzyme.

It appears that POR may play a critical role in the conversion of pyruvate to acetyl-CoA in *H. pylori*, as neither pyruvate dehydrogenase activity nor pyruvate formate-lyase activity has been detected in this organism (13a) and cell suspensions of *H. pylori* have been shown to rapidly oxidize pyruvate to acetate and CO_2 (8). As is characteristic of most POR enzymes, that from *H. pylori* was found to be oxygen sensitive. Omitting dithiothreitol from purification buffers resulted in rapid inactivation of this enzyme. This is interesting in view of the fact that *H. pylori* is a microaerophile rather than an anaer-

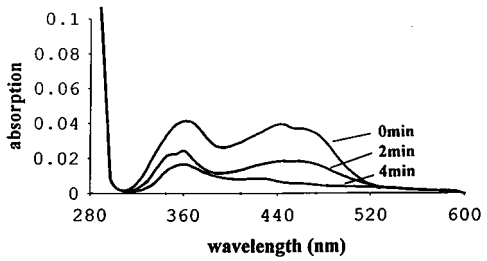

FIG. 5. Spectral characteristics and reduction by POR of *H. pylori* flavodoxin. Flavodoxin was partially purified as described in Materials and Methods. The fully oxidized spectrum is shown at 0 s and exhibits peaks at 375.6 and 452.2 nm. Mono Q partially purified POR was incubated anaerobically with flavodoxin in the presence of 5 mM pyruvate and 0.1 mM CoA. Scans after 2 and 4 min of incubation show quenching of the absorption peaks because of reduction by POR.

TABLE 5. Alignment of *H. pylori* POR N-terminal amino acid sequences

Organism[a]	Subunit	Sequence[b]
H. pylori	47 kDa	AKSIELQEIEVW**DGN**T**A**SNT**L**
A. fulgidus	α	MRKV**VRG**FYSV**A**YSVKLAKPN**V**IXA**YPIT**
T. maritima	α	MERVVERVA**VTG**A**EA**V**A**NAMRQIEPD**V**VA**AYPITPQT**PIV**E**YF
P. furiosus	α	KVMK**GNEA**A**A**W
Anabaena variabilis		**IDGNEA**V**AR**V**A**YKLN-E**VIAIYPITPSS**PMA**E**WS
Anabaena sp. strain PCC 7119		MS-QTFAT**IDGNEA**V**AR**V**A**YKLN-E**VIAIYPITPSS**AMG**E**WA
K. pneumoniae		MSG-KMKTM**DGN**A**AAA**W**I**SYAFT-E**VA**I**YPITP**S**T**PMA**E**NV
Rhodospirillum rubrum		MSVRKMVA**IDGNEA**C**AS**V**A**YRVS-E**VA**V**IYPITPSS**TMG**E**LS
Enterobacter agglomerans		MPG-KMKTM**DGN**T**AA**A**Y**V**S**YAFT-D**VT**AIYPITP**S**T**PMA**E**SV
T. maritima	γ	XIRKVMKA**NEA**A**A**WAAKLAKPK**VIA**AF**P**XX**P**
Halobacterium halobium	α	MTDDELIWR**IAG**GSGDGIDSTSQNFAKALMRSGLDVFTHRHYP
H. pylori	36 kDa	MIKEVKTLKGFSQSAEKFQGS**H**LL**CPGCG**HG**IIV**REV**L**
T. maritima	β	PVNXKQLAQDEFDKKEITQ**GH**RL**XPGXG**AP**ITVK**F**V**M
A. fulgidus	β	MKYFGS**GH**GA**XPGXG**LP**IAVKTV**
P. furiosus	β	AVRKPPITTREYWAP**GH**AA**X**A**GG**
Halobacterium halobium	β	MSKAFSAIDEDREVDRDAFTPGVEPQPTW**CPGCG**DFGV**LK**A**L**K
H. pylori	24 kDa	**M**FQ**IR**W**H**A**RAGQGAIT**GAKG**LA**DVIS**K**T**
A. fulgidus	γ	ML**IEVRFHGRGGQGAVTAA**DL**LA**V**AGFK**
P. furiosus	γ	**MIEV**A**FHGRGGQ**K**AVTAA**N**ILA**EAA**F**LG**
H. pylori	14 kDa	MKDWNEFEMGAV**LF**P**F**EKN**AQSE**ME**K**HN**D**
A. fulgidus	δ	MKIKIN**LGAISE**PM**QSE**NL**K**TG**D**FG
P. furiosus	δ	AESPFK**ADIERAQKE**LS
T. maritima	δ	SLKSWKEI**PI**GGVID**K**PGTA

[a] The following species and/or strains were used: *H. pylori* NCTC 11637 (this paper), *A. fulgidus* VC-16 (30), *T. maritima* (4), *P. furiosus* (3, 4), *Anabaena variabilis* ATCC 29413 (49), *Anabaena* sp. strain PCC 7119 (50), *K. pneumoniae* (6), *Rhodospirillum rubrum* (EMBL accession X77515), *E. agglomerans* 333 (29), and *Halobacterium halobium* (45).

[b] Identical residues and conserved substitutions that align in at least 50% of sequences are indicated by boldface type. Hyphens have been positioned to maximize alignments. X, no phenylthiohydantoin residue identified; probably cysteine.

obe and raises the possibility that some form of in vivo protection against oxygen inactivation may exist. A number of artificial electron acceptors were found to couple with this enzyme, with MV used routinely. It is likely that the in vivo electron acceptor for POR is flavodoxin because of pyruvate- and POR-dependent reduction of a flavodoxin-containing protein fraction. The drug metronidazole, which has a MIC for *H. pylori* of 4 μg ml^{-1} (35a), requires reduction of the nitro group to kill sensitive anaerobes (19). Indirect evidence that *H. pylori* POR can effect this reduction in vitro was obtained in this study, as the addition of metronidazole to the standard assay resulted in a significant lag before MV was reduced. This delay may be due to preferential reduction of the drug before the reduction of MV. However, reduced MV-dependent reduction of metronidazole is more likely, since the midpoint redox potential of the metronidazole couple (−415 mV) (19) is slightly more positive than that of the MV couple (−446 mV) (14). This would result in MV being rapidly reoxidized, giving a delay in development of the colored reduced form until all of the added metronidazole had been reduced.

Our results give further insights into the basic metabolic activities of *H. pylori*. It appears that this organism carries out few primary CO_2 fixation reactions. Kinetic studies of those present may elucidate their role in the CO_2 dependence characteristic of *H. pylori*. Oxidative decarboxylation of pyruvate, a significant reaction in intermediary metabolism, is carried out in *H. pylori* by pyruvate:acceptor oxidoreductase, in place of the aerobic pyruvate dehydrogenase enzyme and pyruvate-formate lyase associated with mixed-acid fermentation. Flavodoxin has been isolated from *H. pylori* extracts and is believed to act as the in vivo electron acceptor for this oxidoreductase. The absence of this enzyme in mammalian systems and the apparent lack of an alternative route for pyruvate conversion in *H. pylori* suggest that this enzyme makes a good target for novel anti-*H. pylori* agents.

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