Characterization of a Novel Bifunctional Dihydropteroate Synthase/Dihydropteroate Reductase Enzyme from *Helicobacter pylori*[▽]

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Tetrahydrofolate is a ubiquitous C₁ carrier in many biosynthetic pathways in bacteria, importantly, in the biosynthesis of formylmethionyl tRNA^{fMet}, which is essential for the initiation of translation. The final step in the biosynthesis of tetrahydrofolate is carried out by the enzyme dihydrofolate reductase (DHFR). A search of the complete genome sequence of *Helicobacter pylori* failed to reveal any sequence that encodes DHFR. Previous studies demonstrated that the *H. pylori* dihydropteroate synthase gene *folP* can complement an *Escherichia coli* strain in which *folA* and *folM*, encoding two distinct DHFRs, are deleted. It was also shown that *H. pylori* FolP possesses an additional N-terminal domain that binds flavin mononucleotide (FMN). Homologous domains are found in FolP proteins of other microorganisms that do not possess DHFR. In this study, we demonstrated that *H. pylori* FolP is also a dihydropteroate reductase that derives its reducing power from soluble flavins, reduced FMN and reduced flavin adenine dinucleotide. We also determined the stoichiometry of the enzyme-bound flavin and showed that half of the bound flavin is exchangeable with the soluble flavins. Finally, site-directed mutagenesis of the most conserved amino acid residues in the N-terminal domain indicated the importance of these residues for the activity of the enzyme as a dihydropteroate reductase.

Tetrahydrofolate (H₄-folate) is a ubiquitous C₁ carrier that is involved in the biosynthesis of thymidine, methionine, pantothenic acid, and purines. In bacteria, H₄-folate is also required for the synthesis of formylmethionyl tRNA^{fMet}, which is essential for the initiation of protein synthesis. The last three steps in the biosynthesis of H₄-folate are the condensation of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate with *para*-aminobenzoate by the enzyme dihydropteroate synthase (DHPS, or FolP) (6), the attachment of one or more glutamic acid residues to the carboxyl end of the *para*-aminobenzoate by the enzyme dihydrofolate synthase (2, 3) (FolC), and finally, the reduction of the dihydrofolate to produce H₄-folate. The last step is performed by the enzyme dihydrofolate reductase (DHFR) (4) that utilizes NADPH as an electron donor. The DHFRs of most organisms are homologues of the enzyme that is encoded by the *Escherichia coli folA* gene. It was recently found that a few bacteria contain an alternative DHFR that belongs to the large family of short-chain dehydrogenases reductases. In *E. coli*, such an alternative DHFR is encoded by the gene *folM* (8). Although H₄-folate is essential for the vital biosynthetic pathways mentioned above, no *folA* or *folM* homologues in the genomes of several bacteria and archaea whose genomes have been fully sequenced could be identified (14), even though these organisms possess genes that use H₄-folate for the production of the metabolites mentioned above.

Recently, we have shown that *Helicobacter pylori* FolP can

complement an *E. coli* mutant whose two functional DHFR genes, *folA* and *folM*, have been deleted (13). Furthermore, we could show that the complementing activity is dependent upon a 100-amino-acid-long N-terminal domain that binds flavin mononucleotide (FMN). Homologues of this domain were also found in several other bacteria and archaea that do not have either *folA* or *folM* homologues. Here we present evidence that *H. pylori* FolP can act as a dihydropteroate reductase, that the electron donor for this enzyme is soluble reduced flavin adenine dinucleotide (FADH₂) or reduced FMN (FMNH₂), and that key conserved amino acids in this N-terminal region are essential for FMN binding.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma. Dihydropteroate was obtained from Schircks Laboratories, Switzerland.

Bacterial strains and growth media. A list of *E. coli* strains and plasmids used in this study is given in Table 1. Bacteria were grown in Luria-Bertani (LB; Difco) medium or M9-CAT medium containing M9 salts, 0.4% (wt/vol) glucose, 10 g/liter Casamino Acids (Difco), and 40 μg/ml thymidine. When indicated, the media were supplemented with the appropriate antibiotic: ampicillin (100 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (170 μg/ml; Sigma). *E. coli* strain DH12S (Invitrogen) was used for all plasmid work.

Construction of *E. coli* strain MM808. To construct *E. coli* strain MM808 ($\Delta thyA \Delta folM$), the *folM* gene was deleted from *E. coli* MM512 (MG1655 $\Delta thyA$) (9) as described in reference 8. Briefly, strain MM512 was transformed with plasmid pMM712 (containing the flanking regions of *folM* cloned into pCVD442) and the transformants were spread onto LB plates containing 5% (wt/vol) sucrose. Candidates for cells carrying the deletion were verified by PCR.

Construction of plasmid pMM902 for expression of the *E. coli fre* gene. The *fre* gene (5) was PCR amplified from genomic DNA of *E. coli* MG1655 by using primers Fre fw and Fre rev. The PCR-amplified fragment was digested with NdeI and HindIII and cloned into the pUC120 vector, which had been modified to contain a His tag coding sequence positioned immediately downstream of the ATG initiation codon, followed by an NdeI site.

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
MM512	MG1655 $\Delta thyA$	9
MM612	MG1655 $\Delta thyA \Delta folA::Kan$	8
MM808	MG1655 $\Delta thyA \Delta folM$	This work
MM847	MG1655 $\Delta folA \Delta thyA \Delta folP::Kan$	13
MM851	MG1655 $\Delta thyA \Delta folM \Delta folA::Kan$ (pMM850)	13
MM902	DH12S(pMM902)	This work
MM1059	MG1655 $\Delta folA \Delta thyA \Delta folM::Kan$ (pFolP-M28E)	This work
MM1060	MG1655 $\Delta folA \Delta thyA \Delta folM::Kan$ (pFolP-K31A)	This work
MM1061	MG1655 $\Delta folA \Delta thyA \Delta folM::Kan$ (pFolP-K92E)	This work
MM1071	MG1655 $\Delta folA \Delta thyA \Delta folM::Kan$ (pFolP-K51E)	This work
Plasmids		
pMM712	<i>folM</i> -flanking regions cloned into pCVD442	8
pMM850	<i>H. pylori folP</i> gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag and a chloramphenicol resistance cassette	13
pMM902	<i>E. coli fre</i> gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag	This work
pFolP-M28E	<i>H. pylori folP</i> M28E mutant gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag and a chloramphenicol resistance cassette	This work
pFolP-K31A	<i>H. pylori folP</i> K31A mutant gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag and a chloramphenicol resistance cassette	This work
pFolP-K51A	<i>H. pylori folP</i> K51A mutant gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag and a chloramphenicol resistance cassette	This work
pFolP-G58A	<i>H. pylori folP</i> G58A mutant gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag and a chloramphenicol resistance cassette	This work
pFolP-K92E	<i>H. pylori folP</i> K92E mutant gene cloned into pUC120 and expressed from the <i>lac</i> promoter with an N-terminal His tag and a chloramphenicol resistance cassette	This work

Introduction of the *H. pylori* FolP point mutations. The introduction of the M28E point mutation was performed by PCR in two steps. In the first step, two PCR products were generated by using the pMM850 plasmid as a template and primers M28ERev and pUC120Fw for one product (240 bp long) and primers M28EFw and BstXIRev for the second product (225 bp long). The two PCR products were purified by agarose gel electrophoresis. In the second step, the two fragments from the first step were fused by PCR by using the pUC120Fw and BstXIRev primers and the 100-fold-diluted purified fragments from the first step as templates. The 465-bp PCR product was cloned into pGEM-T, sequenced, and subcloned into pMM850 by using the restriction enzymes NdeI and BstXI. The resulting plasmid, pFolP-M28E, was resequenced to ensure that the desired mutation was in place. The K31A, K51A, and G58A mutant proteins were generated similarly by using suitable primers (Table 2).

The *H. pylori* FolP K92E mutant protein was created by using pMM850 as a template and primers K92ERev and pUC120Fw. The resulting fragment of 450 bp was cloned into pGEM-T, sequenced, and then subcloned into pMM850 by

using restriction enzymes NdeI and BstXI. The resulting plasmid pFolP-K92E was resequenced to ensure that the desired mutation was in place.

Deletion of the *E. coli folA* gene in a strain carrying wild-type or mutant *H. pylori* FolP. The deletion of the *E. coli folA* gene was performed as previously reported (7, 8). Briefly, the pKD46 plasmid and the pUC120 plasmid carrying the wild-type *H. pylori* FolP or mutated FolP were introduced into strain MM808 ($\Delta folM \Delta thyA$). The *E. coli folA* gene was replaced by a kanamycin resistance gene as follows: a DNA fragment containing a kanamycin resistance gene cassette flanked by approximately 1,000-bp-long fragments of the upstream and downstream regions of the *E. coli folA* gene was PCR amplified by using primers eD5'up and eD3'down and DNA extracted from strain MM612 ($\Delta thyA \Delta folA::Kan$) as the template. The PCR fragment was used to transform strain MM808 carrying the wild-type or a mutated version of the *folP* gene. Kanamycin-resistant colonies were selected, and the pKD42 plasmid was cured by growing the cells at 37°C. The replacement of the *folA* gene was confirmed on LB plates supplemented with 40 µg/ml thymidine and 25 µg/ml kanamycin. Colonies that

TABLE 2. List of primers used for the PCRs and their locations in relation to various genes

Primer name	Sequence (5'→3')	Location, description, and/or starting point
eD-short-up	GTTTACGCTTTACGTATAGTGG	−50 to −31 relative to the <i>folA</i> gene
eD-short-down	GTCGCATCCGGCGCTAGCC	+515 to +497 relative to the <i>folA</i> gene
eD3'down	ATGCATGCGAAACCCGCTGGGCACCATGC	+1519 to +1498 relative to the <i>folA</i> gene
eD5'up	ATTCTAGACGCCATGCTGTGGCTGATTGC	−1131 to −1111 relative to the <i>folA</i> gene
Fre fw	CATATGACAACCTTAAGCTGTAAAGTGACC	5' end of the <i>E. coli fre</i> gene; forward primer
Fre rev	GGATCCTCAGATAAATGAAAACGCATCGC	3' end of the <i>E. coli fre</i> gene; reverse primer
pUC120Fw	GCAACGCAATTAATGTGAGTTAGTCTC	−120 relative to the <i>folP</i> start codon in pMM850; forward primer
BstXIRev	TAAGCGCAAAGGGTTGGGAGTGG	+306 relative to the <i>folP</i> start codon in pMM850; reverse primer
M28ERev	CTTTTGTGGTCTCGCGATCTTGC	+121 relative to the <i>folP</i> start codon in pMM850; reverse primer
M28EFw	GCAAGATCGCGAGCACCACAAAAG	+99 relative to the <i>folP</i> start codon in pMM850; forward primer
K31AFw	GCAACGCAATTAATGTGAGTTAGTCTC	+107 relative to the <i>folP</i> start codon in pMM850; forward primer
K31ARev	GCTAACGCCTGCTTGGTGCATGC	+129 relative to the <i>folP</i> start codon in pMM850; reverse primer
K51AFw	CGCTCATTTTTCGCGCAAGAGGCC	+167 relative to the <i>folP</i> start codon in pMM850; forward primer
K51ARev	GGCCTCTTGCCTGCTAAATGAGCG	+189 relative to the <i>folP</i> start codon in pMM850; reverse primer
G58AFw	CATAAGCGTTGCGGGCGATTTTG	+204 relative to the <i>folP</i> start codon in pMM850; forward primer
G58ARev	CAAAATCGCCGCAACGCTATG	+211 relative to the <i>folP</i> start codon in pMM850; reverse primer
K92ERev	CCAAAGGGTTGGGAGTGGCACTCAAG	+323 relative to the <i>folP</i> start codon in pMM850; reverse primer

grew on the plates were respread onto LB plates supplemented with 40 μ g/ml thymidine and 25 μ g/ml kanamycin, and the deletion was confirmed by colony PCR using primers eD-short-up and eD-short-down.

Determination of growth rates of the mutant strains. Strains harboring the wild-type or a mutant variant of FolP were grown overnight in LB-kanamycin medium, harvested, and washed twice with M9-CAT to remove the remains of the rich medium. All of the strains were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in M9-CAT at a final volume of 3 ml, and the mixture was shaken at 37°C. The optical density was monitored at approximately 45-min intervals for 9 h. Each experiment was repeated three times.

Expression and purification of the Fre recombinant protein. *E. coli* strain MM902 (DH12S harboring pMM902) was cultured with shaking at 37°C in LB medium containing ampicillin to an OD_{600} of 0.6. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the culture was shaken overnight at 37°C. Cells were harvested; suspended in 200 ml of a buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole, pH 8.0; and disrupted by sonication. The supernatant was separated from the cellular debris by centrifugation for 10 min at 10,000 rpm by using a Sorvall SS-34 rotor, and the Fre recombinant protein was purified as follows. The supernatant was loaded onto a 5-ml Ni-CAM resin (Sigma) column that was preequilibrated with a buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole, pH 8.0. The column was washed thoroughly with the same buffer until no further reduction in the optical density at 280 nm was observed. The protein was eluted with a buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 250 mM imidazole, pH 8.0. The purified Fre was dialyzed 10,000-fold against a mixture of 300 mM NaCl, 50 mM sodium phosphate, and 10% glycerol, pH 7.2, and then divided into 100- μ l aliquots and stored at -70°C for future use.

Purification of the recombinant *H. pylori* FolP. The purification of the recombinant *H. pylori* FolP was performed as described in reference 13, with some modifications. In order to stabilize the enzyme, all the buffers used in the purification protocol contained 10% glycerol. For further purification, the histidine-tagged protein that had been eluted from the Ni-CAM (Sigma) was dialyzed 2,500-fold against a buffer containing 400 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 10% glycerol and then concentrated to a final volume of 5 ml by using a Vivaspinn 20-ml concentrator with a 30-kDa cutoff (Vivascience AG, Hanover, Germany). The concentrated FolP was subjected to gel filtration by using a HiPrep 16/60 column, containing Sephacryl S-200 high-resolution medium (Amersham Biosciences), that had been preequilibrated with a buffer containing 10% glycerol, 400 mM NaCl, and 50 mM Tris HCl, pH 8.0. Following gel filtration, the FolP was brought to a final concentration of 12.5 mg/ml by using a Vivaspinn 20-ml concentrator with a 30-kDa cutoff, divided into 50- μ l aliquots, flash frozen in liquid nitrogen, and stored at -70°C for future use.

The M28E, K31A, and K92E mutant FolP proteins were purified as described above with slight modifications that aimed at minimizing inclusion body formation: 250 ml of a culture of an *E. coli* strain ($\Delta folA \Delta folM \Delta thyA$) carrying the mutated FolP was shaken in LB medium at 37°C until the OD_{600} reached 0.6. The culture was brought to 25°C, IPTG was added to a final concentration of 0.05 mM, and the culture was left to shake overnight. The purification steps were performed as described above.

Conversion of dihydropteroate into tetrahydropteroate by *H. pylori* FolP in an oxidative half reaction. *H. pylori* FolP was diluted to a final concentration of 17 μ M in a buffer containing 300 mM NaCl and 100 mM sodium phosphate, pH 8, in an airtight cuvette. After purging of the oxygen, the FolP was reduced by adding aliquots of sodium dithionite until the optical absorption of the bound FMN at 450 nm was nearly zero. Deoxygenated H_2 -pteroate was then added to a final concentration of 1 mM, and the change in the optical absorption was monitored.

FADH₂-dependent reduction of H_2 -pteroate by *H. pylori* FolP in a coupled reaction employing *E. coli* FMN reductase and NADH. A mixture containing 300 μ M NADH, 11.3 to 75 μ M flavin adenine dinucleotide (FAD) in 150 mM NaCl, 25 mM sodium phosphate buffer (pH 8.0), and 1.25% (wt/vol) glycerol was deoxygenated in an airtight cuvette. Deoxygenated FolP and Fre were added to final concentrations of 10.5 μ M and 0.8 μ M, respectively, and the reaction mixture was incubated until the FAD was completely reduced, as judged by the lack of further change in the optical absorbance at 340 nm. Deoxygenated H_2 -pteroate, in the same buffer, was then added to a final concentration of 50 μ M, and the change in the optical absorption at 340 nm was monitored. In a control reaction in which H_2 -pteroate was replaced by buffer, no change in the optical absorption could be detected.

Exchange of the FolP-bound FMN for FAD. The exchange reaction was performed by incubating 250 μ M FAD with FolP bound to 22 μ M FMN in a coupled-reaction mixture that contained 500 μ M NADH, 100 μ M H_2 -pteroate,

and *E. coli* Fre. The reaction mixture was incubated for 30 min under anaerobic conditions. Subsequently, FolP was separated from the excess FAD by using a desalting column (Amersham Biosciences) that had been preequilibrated with 400 mM NaCl–25 mM Tris buffer, pH 8.0, at a flow rate of 4 ml/min. Bound flavin was released from the FolP by adding sodium dodecyl sulfate (SDS) to a final concentration of 0.14%, and the concentration of the released flavin was adjusted to 14 μ M. The fluorescence of the released flavin was determined by measuring the emission spectra at 525 nm following excitation at 450 nm by using an LS50B fluorescence spectrometer (PerkinElmer). As a control, FolP was treated in the same way but without the addition of FAD. The levels of fluorescence of 14 μ M FAD and 14 μ M FMN in a buffer containing 400 mM NaCl and 25 mM Tris, pH 8.0, were measured as standards.

RESULTS

Analysis of conserved residues in the N-terminal domain of *H. pylori* FolP. Previously (13), we have shown that *H. pylori* FolP contains an extra N-terminal domain compared to the *E. coli* homologue and that this domain is responsible for the ability of this enzyme to replace the DHFR activity *in vivo* in *E. coli* mutants that lack both *FolA* and *FolM*. Homologues to this domain in several bacteria and archaea were also found, either in the N or C termini of the organisms' FolP proteins. Using the ClustalW program, we aligned the homologous domain sequences and noticed that some residues were conserved in all the domains, as shown in Fig. 1. In order to test whether the most conserved residues are essential for the reducing activity of *H. pylori* FolP, the residues were mutated and the abilities of the modified FolP enzymes to replace *E. coli* DHFR were determined by testing whether the *folA* gene could be deleted from an *E. coli* $\Delta folM$ strain carrying the mutated *H. pylori* FolP. In those cases in which the FolP mutant proteins enabled the growth of the *E. coli* $\Delta folM \Delta folA$ strain, their effect on the growth rate was determined by comparison to the growth rate of the *E. coli* double mutant harboring the wild-type *H. pylori* FolP.

Mutations in the five most conserved residues, M28E, K31A, K51A, G58A, and K92E, were created as described in Materials and Methods, and the mutant genes were cloned into a modified pUC120 expression vector that contains a region coding for an amino-terminal histidine tag. The various plasmids were introduced into *E. coli* MM808 ($\Delta thyA \Delta folM$), and attempts were made to replace the strain's genomic *folA* gene with a kanamycin resistance cassette by using the one-step gene replacement technique as described in Materials and Methods. Successful replacement of the *folA* gene was verified by PCR as described in Materials and Methods. Four of the FolP mutant proteins were functional, enabling the successful deletion of the *E. coli* *folA* gene, whereas with the FolP G58A mutation, it was impossible to delete *folA*. To assess whether this mutation affected the reducing function of the N-terminal domain or possibly hampered the folding of the entire protein, the ability of the FolP G58A mutant protein to function as a dihydropteroate synthase and to complement a deletion of *folP* in *E. coli* strain MM847 ($\Delta folA \Delta thyA \Delta folP$) was examined. Previously, we showed that the MM847 strain cannot grow on M9-CAT medium, although it can still form colonies on LB rich medium (13). However, when a pUC120 plasmid expressing either the wild-type *H. pylori* FolP or the G58A mutant protein was introduced into MM847, the strain was able to grow on M9-CAT, suggesting that the G58A mutation does not affect the dihydropteroate synthase function of *H. pylori* FolP.

FIG. 1. Alignment of the FolP linker domains. Amino acids identical to the consensus are highlighted in black, and amino acids that are similar to the consensus are highlighted in gray. The arrows indicate where point mutations were introduced. Mutations are listed in boxes next to the arrows. Numbers on the left are amino acid positions. Organisms and NCBI accession numbers corresponding to the homologous proteins are as follows, in order of the appearance of the protein sequences: *Caldicellulosiruptor saccharolyticus* DSM 8903, ZP_00884268.1; *Clostridium thermocellum* ATCC 27405, ZP_00503815.1; *Methanosarcina mazei* Go1, NP_632464.1; *Methanosarcina acetivorans* C2A, NP_618399.1; *Methanosarcina barkeri* strain Fusaro, YP_305818.1; *Methanococcoides burtonii* DSM 6242, YP_566451.1; *Thermoanaerobacter ethanolicus* ATCC 33223, ZP_00778502.1; *Thermoanaerobacter tengcongensis* MB4, NP_623925.1; *Syntrophomonas wolfeii* subsp. *wolfeii* strain Göttingen, YP_752825.1; *Moorella thermoacetica* ATCC 39073, YP_428992.1; *Desulfotobacterium hafniense* DCB-2, ZP_01371638.1; *Desulfuromonas acetoxidans* DSM 684, ZP_01313207.1; *Pelobacter propionicus* DSM 2379, YP_901918.1; *Haloarcula marismortui* ATCC 43049, YP_134954.1; *Halobacterium* sp. NRC-1, NP_279484.1; *Helicobacter pylori* 26695, NP_208024.1; *Wolinella succinogenes* DSM 1740, NP_907857.1; *Helicobacter hepaticus* ATCC 51449, P_860152.1; *Campylobacter jejuni* RM1221, YP_178700.1; *Campylobacter coli* RM2228, ZP_00367318.1; *Campylobacter lari* RM2100, ZP_00369089.1; *Campylobacter upsaliensis* RM3195, ZP_00369940.1; *Thiomicrospira denitrificans* ATCC 33889, YP_394159.1.

Growth properties of the mutant strains. To assess the relative activities of the different *H. pylori* FolP mutant proteins in vivo, *E. coli* strains ($\Delta folA \Delta thyA \Delta folM$) harboring the FolP mutant proteins were grown in M9-CAT and their growth rates were determined. As a control, we used a strain with the same

background that harbored the wild-type *H. pylori* FolP. The experiment was repeated independently three times. While the *E. coli* strain containing the wild-type *H. pylori* FolP had a doubling time of 72 min, the strains containing the FolP M28E, K31A, and K92E mutant proteins had doubling times of 126,

FIG. 2. Absorbance spectra of the wild-type and mutated FolP proteins. The wild-type and the mutated *H. pylori* FolP proteins were diluted with a buffer containing 200 mM NaCl, 50 mM Tris (pH 8), and 5% (wt/vol) glycerol to a final concentration of 10 μ M, and the absorbance spectra were measured continuously. $\blacklozenge$, wild-type protein; $\bullet$, M28E mutant protein; $\blacktriangle$, K92E mutant protein; $\blacksquare$, K31A mutant protein.

193, and 258 min, respectively. The strain containing the FolP K51A mutant protein could not grow in liquid M9-CAT. It was also noticed that cells that harbored the K51A mutant FolP protein very quickly lost viability even when grown on LB agar plates.

Purification of the FolP mutant proteins and spectroscopic analysis. Three of the FolP mutant proteins, the M28E, K31A, and K92E mutant proteins, were purified as described in Materials and Methods. The yields of the three FolP mutant proteins were lower than the yield of the wild-type enzyme due to the tendency of the mutated proteins to form inclusion bodies. Moreover, the soluble mutant proteins were unstable and tended to precipitate after a few days even when kept at 4°C. The yield of the K51A mutant protein was negligible even with modifications to the purification method. The purified mutant proteins were subjected to SDS-polyacrylamide gel electrophoresis and found to be more than 85% pure.

The mutant proteins, unlike the wild-type enzyme, which had a bright yellow color, were colorless. Spectroscopic analyses of wild-type FolP and the M28E, K31A, and K92E FolP mutant proteins revealed that all three mutant proteins no longer contained FMN (Fig. 2). Attempts to reconstitute the mutant enzymes were made by adding FMN. FMN at a concentration of 1 mM was incubated with 2.5 μ M mutant enzymes, followed by rapid passage through a HiTrap desalting column (Amersham). Monitoring of the absorbance and fluorescence spectra of the mutant proteins failed to reveal any bound FMN. It seems likely, therefore, that the conserved amino acids that had been changed participate in the binding of FMN.

Measurement of the stoichiometry of bound FMN. In order to release bound FMN, a measured amount of the holoenzyme was dissolved in a buffer of 6 M guanidine-HCl and 20 mM sodium phosphate, pH 6.5. The unfolded enzyme was filter washed several times through a 10-kDa-cutoff filter (Vivaspin 0.5-ml concentrator; Vivascience AG, Hanover, Germany), and the optical absorption of the unfolded FolP protein was

determined at wavelengths of 276, 278, 279, 280, and 282 nm. The exact concentration of the protein was calculated by using the ProtParam Web tool (<http://www.expasy.org/tools/protparam.html>). To determine the amount of bound FMN, a measured amount of holoenzyme was dissolved in a buffer containing 6 M guanidine-HCl and 20 mM phosphate, pH 6.5, and its optical absorption at 448 nm was measured. The extinction coefficient of FMN at 448 nm in the guanidine-HCl solution was determined to be 10,500 $M^{-1} cm^{-1}$ by dissolving a known amount of FMN in the 6 M guanidine-HCl buffer and measuring its optical absorption at 448 nm. The ratio of the bound FMN to FolP monomers was found to be 0.923, corresponding to a stoichiometry of 1:1.

FMN is bound to FolP very tightly. Several attempts were made to release the FMN from the FolP polypeptide. While the dialysis of FolP against a large excess of its substrate, H_2 -pteroate, did not release the FMN, dialysis against activated charcoal resulted in the discharge of the FMN. However, the apoprotein was unstable and precipitated rapidly. These results suggest that the FMN is bound very tightly to the FolP polypeptide and that bound FMN is important for the structural integrity of the native FolP.

Determination of the reduction potential of the FMN bound to *H. pylori* FolP. The reduction potential of FolP was calculated from the distribution of electrons between the enzyme-bound FMN and a redox indicator dye by employing anaerobic dithionite spectroscopic titrations. The spectra of oxidized and reduced FolP and phenosafranine were measured separately. An isosbestic point in the spectral changes associated with dye reduction at 410 nm was identified. The enzyme absorbance was insignificant at 550 nm, a wavelength at which there were large changes in the absorbance of the dye. Amounts of oxidized FolP and phenosafranine that gave roughly the same absorbance measurements were mixed and then titrated with dithionite until both the enzyme and the dye were fully reduced. The extent of enzyme reduction at 410 nm was monitored, as was the extent of dye reduction at 550 nm. From the titration curves of FolP and phenosafranine, the redox potential of *H. pylori* FolP was calculated to be -277 mV.

Conversion of dihydropteroate into tetrahydropteroate by *H. pylori* FolP in an oxidative half reaction. The known function of FolP is to produce H_2 -pteroate from 6-hydroxymethyldihydropterin-pyrophosphate and *para*-aminobenzoate. However, because FolP complements both *E. coli* $\Delta folA$ and $\Delta folM$ mutations, it should possess a reducing activity as well. Previous attempts to reduce in vitro dihydropteroate to tetrahydropteroate with *H. pylori* FolP by using either NADPH or NADH as an electron donor were unsuccessful (13). In order to demonstrate that reduced *H. pylori* FolP can reduce dihydropteroate to tetrahydropteroate in vitro, FolP was reduced anaerobically as described in Materials and Methods. Subsequent to the reduction of the FolP, a saturating amount of dihydropteroate was added and the change in the optical absorption spectra was monitored (Fig. 3). The kinetics of the half reaction were too fast to monitor by using a spectrophotometer. Since the FolP oxidation was absolutely dependent on the availability of dihydropteroate, these results suggest that FolP is capable of reducing dihydropteroate to tetrahydropteroate in vitro.

FIG. 3. Absorbance spectra of oxidized FolP, reduced FolP, and reduced FolP that underwent an oxidative half reaction with H_2 -pterate. The optical absorption spectrum of oxidized *H. pylori* FolP was determined ($\blacktriangle$). The enzyme was then reduced by dithionite, and the optical absorption spectrum was measured again ($\bullet$). A saturating amount of anaerobic H_2 -pterate solution was added, and the spectrum of the enzyme that underwent an oxidative half reaction was determined ($\blacksquare$). The spectra were corrected for the dilution effect.

Coupled reaction of the *H. pylori* FolP and the *E. coli* FMN reductase. The observations that the reduced form of *H. pylori* FolP can directly convert dihydropterate into tetrahydropterate in vitro and that *H. pylori* FolP can substitute for DHFR activity in vivo suggest that the reducing agent occurs in the bacterial cytoplasm. Since NADH and NADPH could not provide the electrons for this reaction, other electron donors were sought. It was previously shown that in some biological oxidoreductive reactions, for instance, in the case of the bioluminescence of the enzyme luciferase (17), the electron donor is $FADH_2$. To test the possibility that the electron donor for *H. pylori* FolP is a soluble $FADH_2$, the *E. coli* gene coding for flavin reductase, *fre*, was cloned and the enzyme was purified. Fre reduces free FMN and FAD under anaerobic conditions into $FMNH_2$ and $FADH_2$, respectively, by using NADH as an electron donor (5). The following coupled reaction was designed.

The coupled reaction was monitored by following the change in the optical absorbance at 340 nm due to the oxidation of NADH (see Materials and Methods). It was found that in the absence of FolP, there was no change in the optical absorbance at 340 nm and that both soluble $FADH_2$ and $FMNH_2$ could act as electron donors (data not shown). The K_m for $FADH_2$ was determined to be 16 μM , and the k_{cat} was 1.83 min^{-1} . The K_m for H_2 -pterate could not be determined since the enzyme was at its maximal activity already at an H_2 -pterate concentration as low as 11.3 μM . The rate of reduction of H_2 -folate at

FIG. 4. Relative fluorescence intensities of 14 μM FAD (A), 14 μM FMN (B), 14 μM flavins released from FolP (C), 14 μM flavins released from FolP that had been preincubated with 250 μM FAD (D), and 14 μM flavins released from FolP that had been preincubated with a mixture containing 250 μM FAD, 500 μM NADH, 100 μM H_2 -pterate, and *E. coli* Fre (E).

concentrations of 50 to 200 μM was only 20% of the rate of reduction of H_2 -pterate. These results indicate that the preferred substrate for the reaction is dihydropterate. Thus, *H. pylori* FolP can act as a dihydropterate reductase in vitro, in addition to its known function as a dihydropterate synthase. Interestingly, we noticed that this form of FolP, with an N-terminal flavodomain, occurs exclusively in microorganisms that are either microaerophilic or strictly anaerobic, as might be expected because of the highly reductive environment that is needed for this reaction.

Replacement of FolP-bound FMN with $FADH_2$. In the coupled reaction, we showed that the FolP reduces H_2 -pterate to H_4 -pterate by using soluble $FADH_2$. We considered how the electrons are transferred from $FADH_2$ to the H_2 -pterate. This transfer may occur either by the reduction of the tightly bound FMN or by the replacement of the FolP-bound FMN with the soluble $FADH_2$, which would subsequently transfer the electrons to the H_2 -pterate to produce H_4 -pterate. To address this question, attempts were made to replace the bound FMN with free FAD or $FADH_2$. In order to determine the content of the FolP-bound flavin, the difference in the levels of specific fluorescence of soluble FMN and FAD was exploited. While soluble FMN and FAD have approximately the same extinction coefficient, FMN has a five- to sixfold-higher level of fluorescence. The ability of soluble $FADH_2$ to replace the bound FMN in the FolP-Fre coupled reaction was investigated. Similarly, the ability of free oxidized FAD to replace the FolP-bound FMN was also tested. Full details of the experiments are described in Materials and Methods. From the levels of fluorescence of the flavins released from the enzyme by SDS, it was possible to calculate the amounts of FMN and FAD bound to the enzyme following the exchange reaction. The fluorescence intensity of the soluble flavins released from enzyme that had been preincubated either with FAD or with $FADH_2$ and H_2 -pterate was only 57% of the fluorescence intensity of the flavin released from untreated FolP (Fig. 4). From this result, it is possible to calculate that the bound FolP protein that had been preincubated with either FAD or $FADH_2$ included approximately 50% FAD and 50% FMN. Thus, in about half of the flavin binding sites of FolP, the bound FMN can be exchanged for FAD.

DISCUSSION

The studies reported here establish that *H. pylori* FolP is a bifunctional fusion protein. Besides its previously known activity as a dihydropteroate synthase, it also reduces dihydropteroate to tetrahydropteroate. We propose, therefore, to rename it dihydropteroate synthase/dihydropteroate reductase, or DHPS/DHPR. *H. pylori*, and presumably other microorganisms that contain a FolP protein with a dihydropteroate reductase domain, is likely to produce H₄-folate from H₄-pteroate by using FolC. Interestingly, such microorganisms contain instead of the more commonly *thyA*-encoded thymidylate synthase an alternative flavin-dependent thymidylate synthase (9, 13). This enzyme, ThyX, functions to transfer the methylene group from methylene-H₄-folate without the oxidation of the H₄-folate. Moreover, because the oxidation state of H₄-folate is also unchanged in other H₄-folate-dependent C₁ transfer reactions, *thyX*-containing organisms do not need a DHFR activity. Given that *H. pylori* FolP is involved solely in the de novo synthesis of H₄-folate, whereas *E. coli* FoaA is also required for the recycling of the H₂-folate generated by dTMP production by ThyA, it is not surprising that the k_{cat} for *H. pylori* FolP is about 100-fold lower than that for *E. coli* FoaA.

The *H. pylori* N-terminal domain represents a new class of FMN binding domains that have no sequence homology to other FMN binding domains. Mutations in five of the most conserved residues in the N-terminal domain, M28, K31, K51, G58, and K92, affected the binding affinity of the mutant enzymes for FMN and the ability of the mutant enzymes to complement a deficiency in DHFR. The findings that the mutant enzymes are soluble, though unstable, and that even the wild-type enzyme becomes unstable when its tightly bound FMN is dissociated by charcoal suggest that these residues are involved in the binding of FMN and that the integrity of the native structure depends on the ability of the enzyme to bind the cofactor.

The mechanism by which *H. pylori* FolP carries out reduction in vivo is still unknown. *H. pylori* FolP can reduce in vitro dihydropteroate to tetrahydropteroate by using free FADH₂ or FMNH₂ as an electron donor. However, since the intracellular concentrations of free FAD and FMN are unknown, it is not clear whether they are the actual electron donors in vivo. The only report on the intracellular free flavin concentration in bacteria (16) presents data for *Amphibacillus xylanus*, in which the concentration of free FAD was found to be 13 μ M. Assuming that the concentration of free flavin in *H. pylori* is similar, and taking into account that *H. pylori* grows in semi-anaerobic environments that have low intracellular oxidation potentials, it is reasonable to suppose that the intracellular level of free FADH₂ is close to 16 μ M, which is the K_m for FADH₂ bound to *H. pylori* FolP.

The use of free reduced flavins as electron donors requires the existence of a soluble flavin oxidoreductase that recycles the oxidized flavins back to their reduced form. Indeed, many microorganisms, including *H. pylori*, that possess the flavin binding domain in FolP were shown to contain open reading frames that were classified as corresponding to flavin oxidoreductase activity. An alternative route for electron donation could occur via a flavin reductase that transfers reduced FMN directly to FolP. Enzymes of this class are known for

their ability to form complexes with flavoproteins and to transfer FMNH₂ directly. This type of transfer occurs in the luminous bacterium *Vibrio harveyi*, in which NADPH-dependent flavin reductase transfers FMNH₂ directly to luciferase (12). Given that it was possible to exchange 50% of the bound FMN without the addition of the oxidoreductase, this route is unlikely.

Studies performed with *Staphylococcus aureus* FolP (10) have shown that this dimeric enzyme operates a half-sites reactivity. If the same mechanism holds for *H. pylori* FolP dihydropteroate synthase activity, the fact that only 50% of the bound FMN was exchanged for either FAD or FADH₂ in the presence of H₂-pteroate suggests that indeed only half of the flavin binding sites are involved in the reducing activity.

To date there are four kinds of dihydrofolate/dihydropteroate reductases: type I, the canonical DHFR that is represented by the protein encoded by *E. coli* folA; type II, the plasmid-encoded R67 DHFR (11); the protein encoded by *E. coli* folM-ydgB; a short-chain dehydrogenase reductase class DHFR; and the *H. pylori* FolP DHPR that bypasses the need for DHFR. Remarkably, the four enzymes are structurally unrelated. We have observed that several microorganisms whose entire genome sequences have been determined lack all four types of dihydrofolate reductases. A case in point is the *Streptomyces* species. This leads us to predict the existence of more classes of dihydrofolate/dihydropteroate reductase isoenzymes. Considering the fact that H₄-folate biosynthesis is a well-documented target for antibacterial drugs and that some pathogenic bacteria possess the novel DHPS/DHPR enzyme for H₄-folate synthesis, this pathway may potentially serve as a new target for the development of novel antibacterial drugs.

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