

Article

Crystal Structure and Sequence Analysis of N⁵, N¹⁰-Methylenetetrahydrofolate Dehydrogenase/Cyclohydrolase Enzyme from *Porphyromonas gingivalis*

Sehyeok Im ^{1,2,†}, Hackwon Do ^{1,2,†}, Jisub Hwang ^{1,2} , Youn-Soo Shim ^{3,*}  and Jun Hyuck Lee ^{1,2,*} 

¹ Research Unit of Cryogenic Novel Material, Korea Polar Research Institute, Incheon 21990, Republic of Korea; alex0127@kopri.re.kr (S.I.); hackwondo@kopri.re.kr (H.D.); hjsub9696@kopri.re.kr (J.H.)

² Department of Polar Sciences, University of Science and Technology, Incheon 21990, Republic of Korea

³ Department of Dental Hygiene, Sunmoon University, Asan 31460, Republic of Korea

* Correspondence: ysshim@sunmoon.ac.kr (Y.-S.S.); junhyucklee@kopri.re.kr (J.H.L.); Tel.: +82-41-530-2740 (Y.-S.S.); +82-32-760-5555 (J.H.L.); Fax: +82-41-530-2726 (Y.-S.S.); +82-32-760-5509 (J.H.L.)

† These authors contributed equally to this work.

Abstract: The methylenetetrahydrofolate dehydrogenase–cyclohydrolase (FolD) enzyme has a dual activity of N⁵,N¹⁰-methylenetetrahydrofolate dehydrogenase and cyclohydrolase. This enzyme plays a critical role in the chemical modification of tetrahydrofolate, which is an important coenzyme involved in the synthesis of DNA, RNA, and amino acids. Therefore, bacterial FolD has been studied as a potential drug target for the development of antibiotics. Here, we determined the crystal structure of FolD (PgFolD) from the oral pathogen *Porphyromonas gingivalis* at 2.05 Å resolution using the molecular replacement method. The crystal structure of PgFolD was successfully refined to a crystallographic R-factor of 21.4% (R_{free} = 23.8%). The crystals belong to the space group of P₄₃22 with the unit cell parameters of a = 110.7 Å, b = 110.7 Å, and c = 69.8 Å, containing one subunit in the asymmetric unit. Our analytical size-exclusion chromatography results indicated that PgFolD forms a stable dimer in solution. Additionally, structural and sequence comparison studies with previously known FolDs revealed that PgFolD has a different substrate-binding site residue composition. These findings provide valuable insights for the structure-based development of specific inhibitors against the *Porphyromonas gingivalis* pathogen.

Keywords: crystal structure; drug target; PgFolD; X-ray crystallography



Citation: Im, S.; Do, H.; Hwang, J.; Shim, Y.-S.; Lee, J.H. Crystal Structure and Sequence Analysis of N⁵, N¹⁰-Methylenetetrahydrofolate Dehydrogenase/Cyclohydrolase Enzyme from *Porphyromonas gingivalis*. *Crystals* **2023**, *13*, 1489. <https://doi.org/10.3390/cryst13101489>

Academic Editor: Ki Hyun Nam

Received: 5 October 2023

Revised: 12 October 2023

Accepted: 12 October 2023

Published: 13 October 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Tetrahydrofolate (THF) and THF derivatives, including 5-methyl-THF, 5,10-methylene-THF, and 10-formyl-THF, serve as co-factors in various biological reactions for DNA synthesis, amino acid metabolism, and other critical cellular processes [1–5]. THF is a folic acid compound modified by adding four hydrogen atoms. Various enzymes are involved in the chemical modification of THF. Methylenetetrahydrofolate dehydrogenase–cyclohydrolase (FolD) is a dual-function enzyme that converts N⁵, N¹⁰-methylene-THF into N¹⁰-formyl-THF in a two-step reaction. The first step is the NADP⁺- or NAD⁺-dependent oxidation of N⁵, N¹⁰-methenyl-THF by FolD via its methylenetetrahydrofolate dehydrogenase activity, and the second step is the hydrolysis of N⁵, N¹⁰-methenyltetrahydrofolate into N¹⁰-formyl-THF via the cyclohydrolase activity [6]. FolD knockout studies have shown that it is essential in both Gram-positive and Gram-negative bacteria [7–9].

To date, several bacterial FolDs have been characterized both biochemically and structurally. The crystal structures of the apo form of FolD from *Escherichia coli* (EcFolD; PDB codes 5O28 and 1B0A) have been previously determined [10,11]. Using structural information, methylene-tetrahydrofolate- and NADP-bound structures were modeled, and

key amino acid residues important for substrate and co-factor binding were predicted [11]. In 2012, the crystal structure of FolD from *Acinetobacter baumannii* (AbFolD) was determined. The study report included three crystal structures of AbFolD in a binary complex with NADP (PDB code 4B4U), a ternary complex with NADP and an inhibitor (PDB code 4B4V), and a ternary complex with NADH and another inhibitor named LY354899 (PDB code 4B4W) [11]. The inhibitors used in this study mimicked the intermediate reaction products of this enzyme. Complex structures with two different inhibitors allowed for a more detailed elucidation of the enzyme reaction mechanism of AbFolD [12]. The crystal structure of unliganded FolD from *Pseudomonas aeruginosa* (PaFolD) was also determined in 2012. The loop region (residues 231–243) of the PaFolD structure shows a different conformation than that of the EcFolD and AbFolD structures [12]. Recently, the complex crystal structure of EcFolD-carolacton was determined (PDB code 5O22) [10]. The authors identified the key residues involved in carolacton binding and resistance in *E. coli*. They also suggested the possibility of using carolacton as an anticancer drug because it inhibits the human methylenetetrahydrofolate dehydrogenase/cyclohydrolase enzyme hSMTHFD2, which is overexpressed in cancer cells [10]. Other studies have also shown that FolD is a potential drug target [13,14].

Our research group is interested in the identification and development of effective antibacterial drugs against oral pathogens. *Porphyromonas gingivalis* is an anaerobic, non-motile, Gram-negative bacterium that causes chronic periodontitis [15]. Chronic infection of this pathogen has also been linked to rheumatoid arthritis, heart disease, diabetes, Alzheimer's disease, and other systemic diseases [16–20]. Various antibiotics have been used to prevent infection of *P. gingivalis*. However, since the late 20th century, there have been several cases of acquired resistance to some antibiotics, including amoxicillin, metronidazole, macrolides, tetracyclines, and fluoroquinolones [21–24]. Considering that the resistance against antibiotics that are still available can be acquired through the mutation of the target site, it is essential to develop novel target bioactive molecules [21]. In this study, the FolD-encoding gene of *P. gingivalis* was identified and cloned as a target protein.

To obtain structural information, we expressed, purified, and crystallized the PgFolD protein from *P. gingivalis*. The crystal structure of substrate-free PgFolD was determined at a resolution of 2.05 Å. Multiple sequence alignments and comparative structural studies of PgFolD and other bacterial FolD homologs were performed to understand the differences and characteristics of the active sites of PgFolD. Considering that the species-specific treatment of growth inhibitors is crucial to preserve the beneficial oral bacteria, these new findings from the sequence and structural analysis will provide a better understanding of structure-based drug development against specific oral pathogenic bacteria such as *P. gingivalis*.

2. Materials and Methods

2.1. Protein Expression and Purification

The recombinant plasmid encoding PgFolD was produced by ligating synthesized PgFolD gene fragments (Bioneer, Daejeon, Republic of Korea) into the pET28a vector using *Nde*I and *Xho*I restriction enzymes. The generated plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression analyses. A single colony of cells was cultured in 4 L of Luria–Bertani (LB) medium with 50 µg/mL kanamycin and incubated at 150 rpm and 37 °C until the optical density reached 0.4 at 600 nm. Thereafter, 1 mM isopropyl β-D-1-thiogalactopyranoside was added to the cell culture to induce protein overexpression, and the cells were grown overnight in a shaking incubator (25 °C, 150 rpm). The cell culture was then harvested by centrifugation at 6000 rpm and 4 °C for 20 min. Harvested cell pellets were resuspended in cell lysis buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, and 5 mM imidazole) and disrupted by ultrasonication (Vibra-Cell™, Sonics & Materials, Inc., Newtown, CT, USA). The disrupted cells were centrifuged at 16,000 rpm and 4 °C for 50 min, after which the protein-containing supernatant was separated from cell debris.

As our target protein was tagged with a poly-histidine tag, Ni-NTA agarose resin was used for purification. First, the resin was packed vertically in a column using gravity. After packing, the column was washed with distilled water and equilibrated with cell lysis buffer. The separated supernatant was loaded onto a column and bound to the Ni-NTA resin at a flow rate of 3 mL/min. Weakly bound proteins were washed with washing buffer (30 mM imidazole, 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl). Poly-histidine-tagged PgFold was eluted using an elution buffer (300 mM imidazole, 20 mM Tris-HCl pH 8.0, and 200 mM NaCl). The eluted protein was treated with 40 U of thrombin to remove the poly-histidine tag and incubated using an inverting machine at 4 °C overnight. Final purification was performed by size-exclusion chromatography using a HiLoad Superdex 200 pg column (Cytiva, Marlborough, MA, USA) and fresh buffer without imidazole (20 mM Tris-HCl pH 8.0 and 200 mM NaCl). Purified protein was concentrated to 30 mg/mL in a volume of 5 mL using an Amicon ultrafiltration filter unit with a 10 kDa cutoff value.

2.2. Crystallization and Data Collection

Purified protein was crystallized using the sitting drop vapor diffusion method in an MRC 96-well sitting drop plate (Molecular Dimensions, Rotherham, UK) at room temperature. For primary screening, 300 nL of purified protein (30 mg/mL) was mixed with 300 nL of crystallization screening solution using a ‘Mosquito’ crystallization robot (SPT Labtech, Melbourn, UK). The commercial crystallization suites, including MCSG 1T~4T (Anatrace, Maumee, OH, USA), The PGA Screen, and JCSG Plus (Molecular Dimensions, Rotherham, UK), were used for screening. For solutions in which crystals were formed, the crystallization conditions were further optimized to increase the size of the crystals using the hanging drop diffusion method. The final crystals for X-ray diffraction were obtained under the conditions of 0.1 M Na₂HPO₄/KH₂PO₄ (pH 6.2) with 35% (*w/v*) MPD.

A single crystal was briefly incubated in a reservoir solution at a final glycerol concentration of 20% for cryoprotection. Thereafter, the crystal was mounted on the synchrotron facility beamline 5C (BL-5C) at the Pohang Accelerator Laboratory (PAL, Pohang, Republic of Korea), and complete diffraction images were captured at 1° oscillations per frame using an Eiger 9M detector (Dectris, Baden, Switzerland). The collected data were processed using the HKL-2000 software (HKL Research Inc., Charlottesville, VA, USA) [25].

2.3. Structure Determination and Oligomerization

We used the CCP4 and Phenix suite [26] and Coot software [27] to build the PgFold structure. The initial phasing problem was solved by the molecular replacement method [26]. A template structure from the AlphaFold database (model number AF-Q7MVE9-F1) was used [28]. Iterative refinement was performed using Refmac [29], phenix.refinement [30], and Coot [27]. The refined structure was validated using Molprobit [31,32]. The final structure was deposited in the Protein Data Bank under the accession code (PDB code PgFold: 8WFC). The X-ray diffraction data and refinement statistics are summarized in Table 1.

Size-exclusion chromatography (SEC) was performed using a HiLoad Superdex 200 pg column (Cytiva) at a flow rate of 0.5 mL/min. We used Protein Standard Mix 15–600 kDa (Sigma-Aldrich, Burlington, MA, USA) containing Thyroglobulin bovine (670 kDa), γ -globulins from bovine blood (150 kDa), ovalbumin (44.3 kDa), ribonuclease A type I-A (13.7 kDa), and p-aminobenzoic acid (137 Da) to make a trend curve showing the relationship between the molecular weight of protein and elution volume (Column volume). PgFold was eluted at a flow rate of 0.5 mL/min using a protein storage buffer (20 mM Tris-HCl pH 8.0 and 200 mM NaCl). After confirming that PgFold exists as an oligomer in an aqueous environment through SEC, its dimeric structure was analyzed and visualized using PyMOL v4.6 [33].

Table 1. X-ray diffraction data collection and refinement statistics.

Data Set	PgFolD
X-ray source	BL-5C (PAL, Pohang)
Space group	P4 ₃ 22
Unit cell parameters (Å, °)	a = 110.68, b = 110.68, c = 69.78, α = β = γ = 90
Wavelength (Å)	0.9796
Resolution (Å)	50–2.05 (2.09–2.05)
Total reflections	723,199 (1739)
Unique reflections	27,821 (1347)
Average I/σ (I)	59.67 (10.38)
R _{merge} ^a	0.100 (0.304)
Redundancy	26 (26.3)
Completeness (%)	99.9 (99.9)
Refinement	
Resolution range (Å)	35.01–2.05 (2.12–2.05)
No. of reflections of working set	27,773 (2576)
No. of reflections of test set	1413 (142)
No. of amino acid residues	285
No. of water molecules	163
R _{cryst} ^b	0.215 (0.254)
R _{free} ^c	0.238 (0.288)
R.m.s. bond length (Å)	0.011
R.m.s. bond angle (°)	1.13
Average B value (Å ²) (protein)	40.82
Average B value (Å ²) (solvent)	43.49
Ramachandran plot	
Favored (%)	94.66
Allowed (%)	5.34
Outliers (%)	0.00

^a $R_{\text{merge}} = \sum |<I> - I| / \sum <I>$. ^b $R_{\text{cryst}} = \sum ||F_o| - |F_c|| / \sum |F_o|$. ^c R_{free} calculated with 5% of all reflections excluded from the refinement stages using high-resolution data. Values in parentheses refer to the highest-resolution shells.

3. Results and Discussion

3.1. Overall Structure of PgFolD

The genome of *Porphyromonas gingivalis* strain ATCC BAA-308/W83 was sequenced, and all gene sequence information is available [34]. The sequence UniProtKB ID Q7MVE9, corresponding to the gene encoding PgFolD, was selected for cloning, expression, and purification. Purified PgFolD was successfully crystallized using the hanging drop vapor diffusion method at 293 K. The crystal structure of the apo form of PgFolD was determined at 2.05 Å resolution using the molecular replacement method, with refinement results being 21.5% of R_{factor} and 23.8% of R_{free} . The final model was composed of 285 amino acids and 163 water molecules (Table 1). The overall structure of PgFolD was divided into N-terminal and C-terminal domains. The N-terminal domain has three parallel β-strands (β1–β3), surrounded by six α-helices (α1, α2, α3, α4, α5, and α6), whereas the C-terminal domain contains two separated β-sheets (β4–β7 and β8–β9) and is surrounded by six α-helices (α6, α7, α8, α9, α10, and α11) (Figure 1). The co-factor and substrate-binding sites were located between the N-terminal and C-terminal domains. One protomer of PgFolD was observed in the asymmetric unit, and the Matthews coefficient was 3.35 Å³, with a solvent content of 63.26% [35]. Analytical gel filtration results showed that PgFolD formed a stable dimer in solution. The dimer structure of PgFolD can be generated by crystallographic two-fold symmetry and is very similar to previously known dimeric structures from other bacterial FolD homologs. An accessible surface of approximately 1416 Å² per monomer is buried upon dimerization, accounting for approximately 10.4% of the total monomer surface. The majority of the dimer interface occurs between α5 and β5 of each subunit (Figure 2).

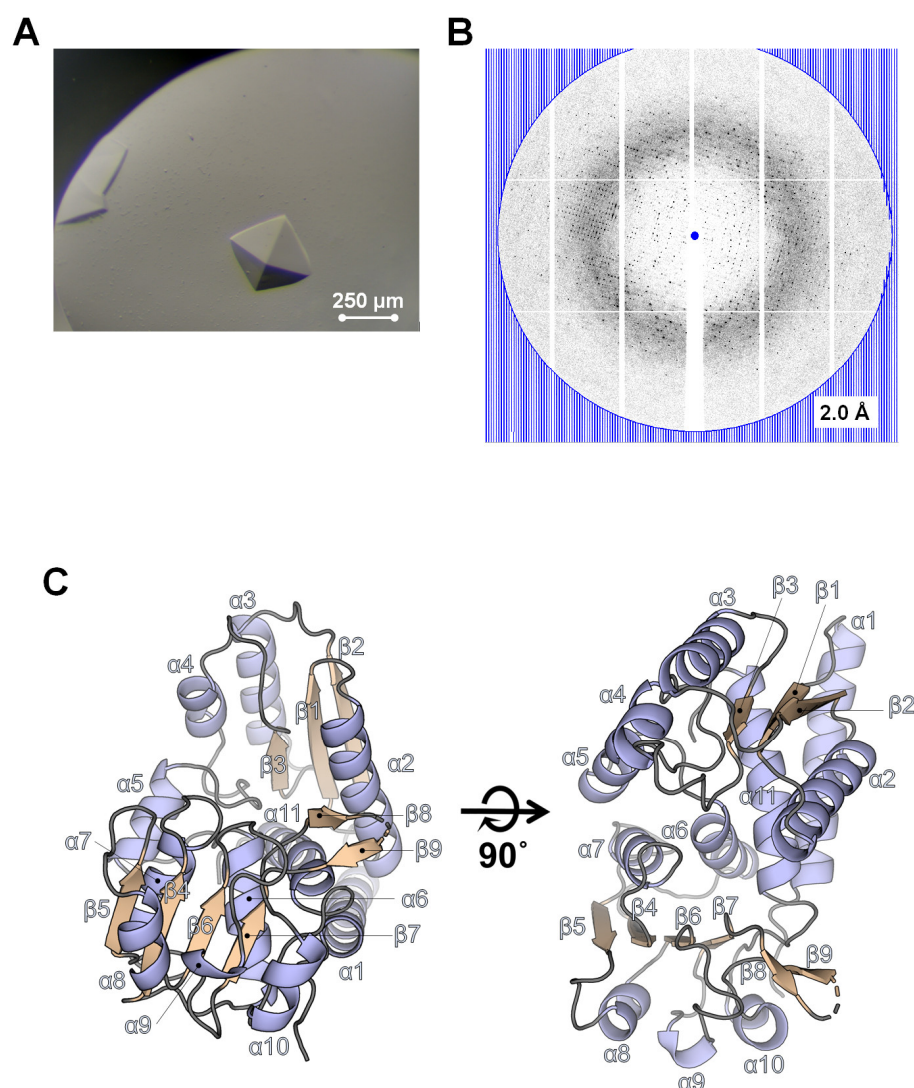


Figure 1. Crystallization, X-ray diffraction, and structure determination of *PgFold*. (A) Crystals of *PgFold* in 96-well sitting drop plate. Scale bar, 250 μm. (B) X-ray diffraction results of *PgFold*. The diffraction data were collected up to 2.05 Å. (C) The overall structure of *PgFold* is shown as a ribbon diagram with 11 α-helices colored light blue and 9 β-stands colored gold.

3.2. Active Site of *PgFold*

To investigate and compare the active site residues in *PgFold*, the apo-*PgFold* structure was superimposed onto the NADP-bound *AbFold* (PDB code 4B4U) and the inhibitor (LY354899)-NADP-bound *AbFold* (PDB code 4B4V) structures that have been reported previously [12]. Among the bacterial Fold structures, the *AbFold* structure was selected for comparison with the *PgFold* structure because both coenzyme- and inhibitor-bound *AbFold* structures are available (Table 2). The amino acid sequence identity between *PgFold* and *AbFold* is 42.7% (Figure 3). Furthermore, we could determine the NADP-binding residues of *PgFold* upon comparing the apo-*PgFold* structure with the NADP-bound *AbFold* structure (PDB code 4B4U) (Figure 4). The NADP-interacting residues of *PgFold* and *AbFold* were very similar, except for Q218 in *PgFold*, corresponding to the K211 in *AbFold*. When the apo-*PgFold* structure was compared to the inhibitor (LY354899)-NADP-bound *AbFold* (PDB code 4B4V) structure, the putative ligand-binding residues of *PgFold* were identified. Through this structural comparison, we could identify different residues in the putative substrate-binding site of *PgFold* compared to that in *AbFold*. *PgFold* has three unique residues (S55, L101, and T238) at the substrate-binding site, whereas *AbFold* has M52, H98, and F231 residues at the corresponding positions (Figure 4D). The distinct

arrangement of residues surrounding the inhibitor binding site suggests that the inhibitor (LY354899) is ineffective in inhibiting the activity of *PgFolD*. This implies the necessity of developing a customized inhibitor specifically designed for *PgFolD* based on the structure information. In addition, the domain-closing movement of *PgFolD* and *AbFolD* by the binding of co-factors and/or inhibitors could be observed through these structural comparison studies. The unliganded form of *PgFolD* contains an open conformation, and NADP binding induces domain closure. When both NADP and the inhibitor were bound to *AbFolD* at the same time, *AbFolD* had the most fully closed conformation (Figure 4E). Next, the surface charges of bacterial apo-FolD structures were investigated and compared. The NADP-binding site exhibited a similar shape and was dominated by a negatively charged electrostatic surface, whereas the surface charge distribution and shape of the substrate-binding site were considerably different (Figure 5). This suggests that it is possible to produce an inhibitor specific to *P. gingivalis* using the structural information of the substrate-binding site.

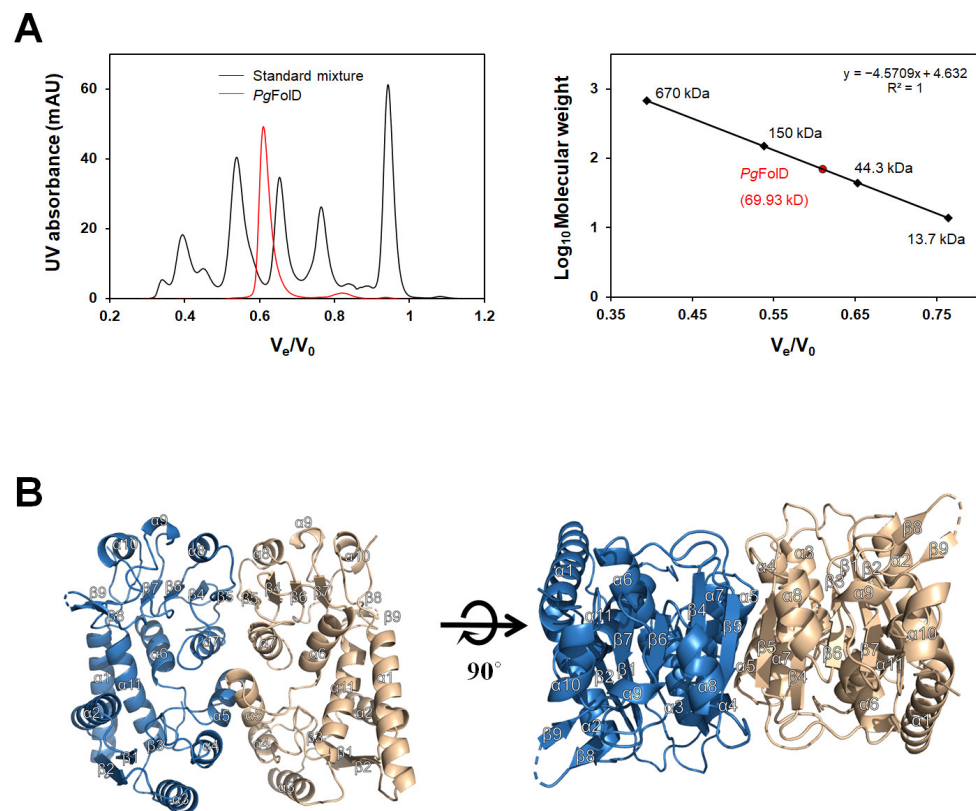


Figure 2. Size-exclusion chromatography results and dimeric structure of *PgFolD*. **(A)** The black curve with five peaks represents the size-exclusion chromatography result of Protein Standard Mix, and the red curve with a single peak represents the result of *PgFolD*. The results show the oligomeric state of *PgFolD* in aqueous solution. **(B)** Dimeric structure of *PgFolD* and its secondary structure. Each molecule of *PgFolD* in the dimeric structure is shown in two different colors (sky blue and wheat).

Table 2. Known bacterial FoLD structures.

Protein Name	PgFolD	AbFolD			EcFolD		PaFolD
Source	<i>Porphyromonas gingivalis</i>	<i>Acinetobacter baumannii</i>			<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>
UniProtKB code	Q7MVE9	D0CBC8			P24186		Q9I2U6
Sequence identity/similarity (%) with PgFolD		39%/57%			44%/59%		45%/60%
PDB code		4B4U	4B4V	4B4W	5O28, 1B0A	5O22	4A5O
Complexed ligand	none	NADP	NADP and inhibitor LY354899	NADP and an inhibitor	none	carolacton	none
Domain conformation	open	closed	closed	closed	open	open	open

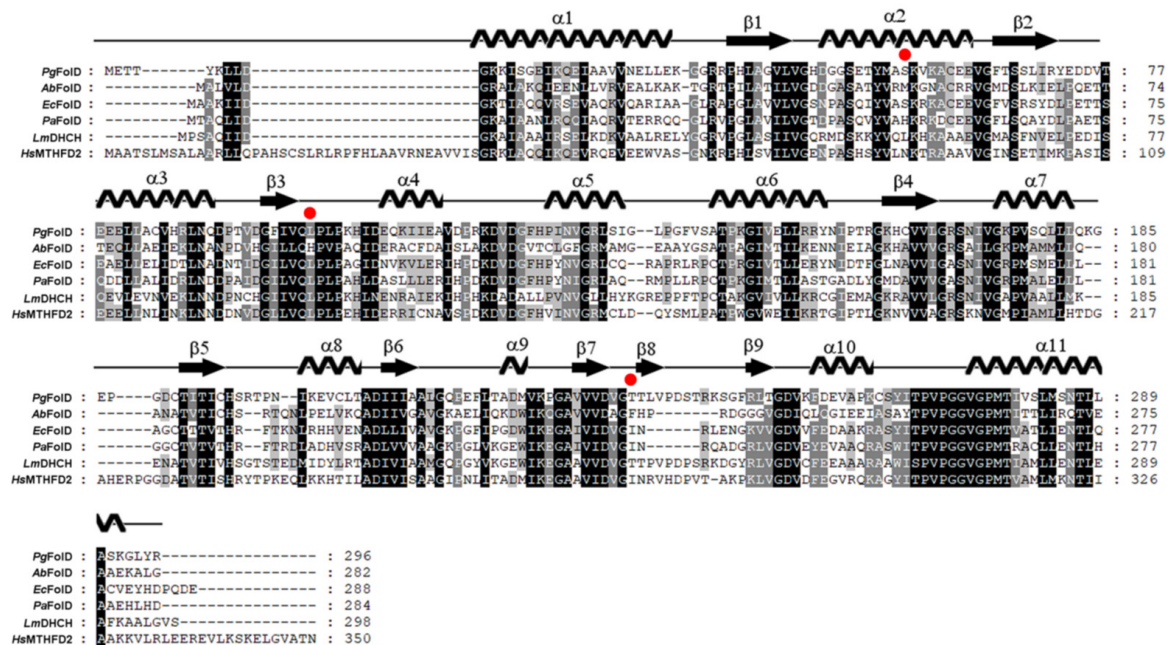


Figure 3. Multiple sequence alignment with other structural homologs. Multiple amino acid sequence alignment was performed between PgFolD (UniProtKB code Q7MVE9) and other FolDs, namely FolD from *Acinetobacter baumannii* (AbFolD; UniProtKB code D0CBC8; PDB codes 4B4U, 4B4V, and 4B4W), *Escherichia coli* (EcFolD; UniProtKB code P24186; PDB codes 5O28, 1B0A, and 5O22), and *Pseudomonas aeruginosa* (PaFolD; UniProtKB code Q9I2U6; PDB code 4A5O), methylenetetrahydrofolate dehydrogenase/cyclohydrolase from *Leishmania major* (LmDHCH; UniProtKB code Q4Q9F9; PDB code 4A26), and bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase from *Homo sapiens* (HsMTHFD2; UniProtKB code P13995; PDB codes 7EHJ, 6KG2, 5TC4). When comparing PgFolD and AbFolD, three residues (S55, L101, and T238) of PgFolD that might be important for substrate binding but are different from the corresponding residues of AbFolD are marked using red circles.

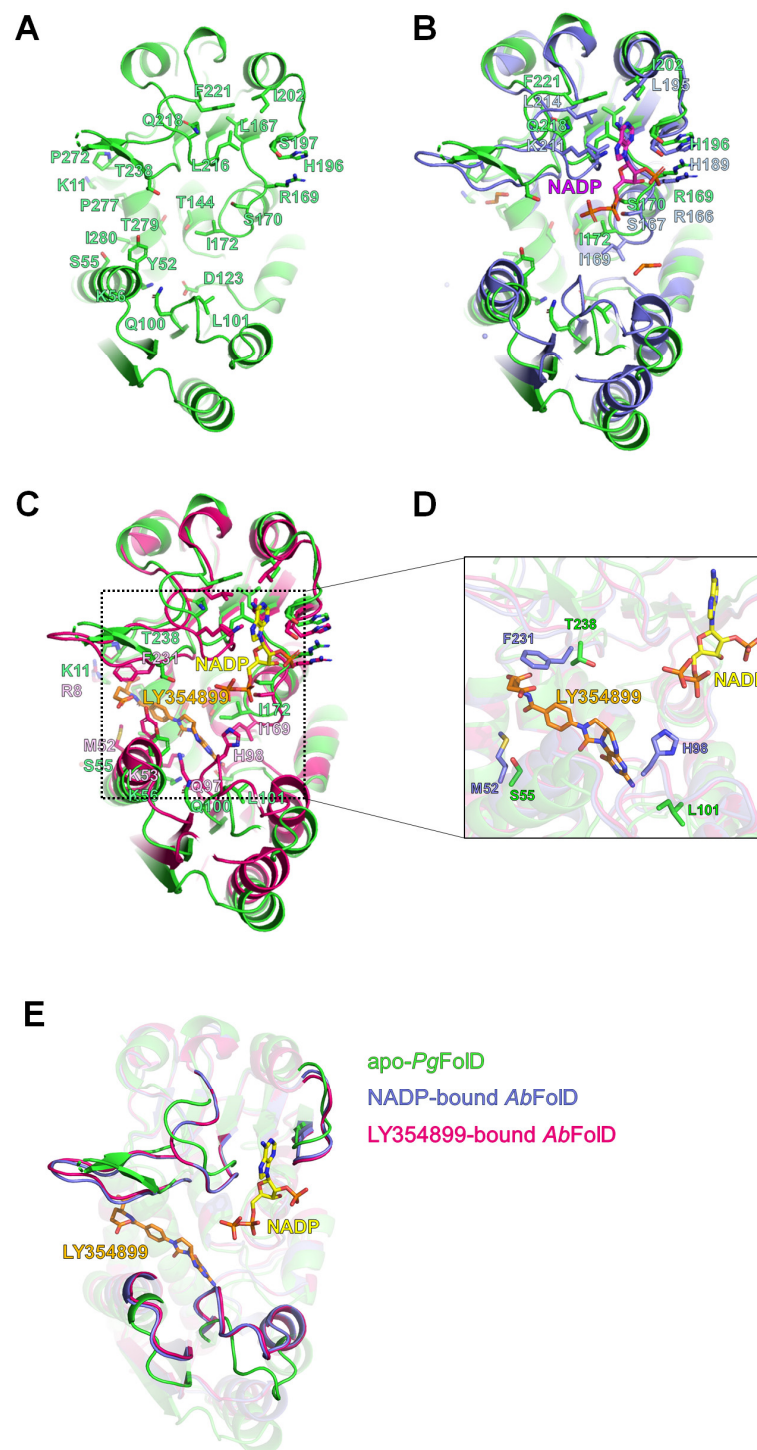


Figure 4. Structural comparison of *PgFold* and *AbFold*. (A) The key residues of *PgFold* involved in the substrate and NADP binding are shown using a stick model. (B) Superimposed structures of *PgFold* (green) and NADP (orange)-bound *AbFold* (slate blue) (PDB code 4B4U). (C,D) Superimposed structures of *PgFold* (green) and inhibitor (LY354899) (orange) NADP (yellow)-bound *AbFold* (red) (PDB code 4B4V). Notably, there are three residues (S55, L101, and T238) in the substrate binding site of *PgFold* that are different from the substrate binding residues in *AbFold*. (E) Structural comparison of apo-*PgFold* (green) and two different *AbFold* structures (PDB codes 4B4U and 4B4V) shows that the apo structure of *PgFold* (green) has open conformation, while the binary (slate blue) and ternary (red) complexed *AbFold* structures have closed conformation.

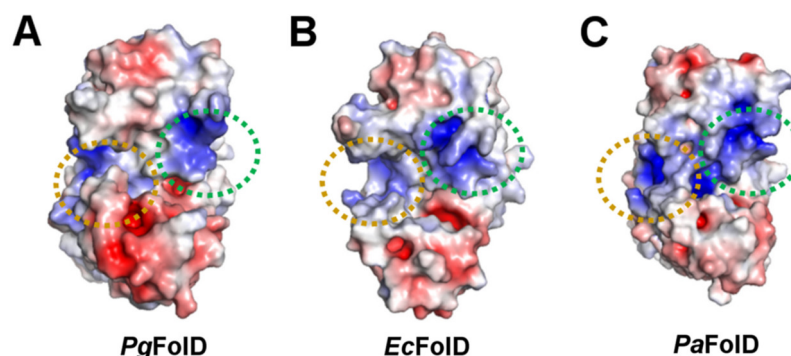


Figure 5. Comparison of electrostatic potentials on surfaces of bacterial apo-Fold structures. Electrostatic surface representation of (A) apo-PgFold, (B) apo-EcFold (PDB code 5O28), and (C) apo-PaFold (PDB code 4A5O) is shown. The NADP and substrate binding sites are indicated using green and gold dotted circles, respectively. Electrostatic surface potential was calculated with APBS software and colored using a calculated charge from red (-5 kT/e) to blue ($+5$ kT/e).

4. Conclusions

In the current study, we described the determination of the apo-form structure of PgFold at a resolution of 2.05 Å. PgFold structure has high overall structural similarity with previously known bacterial Folds. However, multiple sequence alignment and comparative structural analysis revealed that PgFold has unique substrate-binding residues, indicating that PgFold has different binding modes for substrates. While it is necessary to validate the importance of these residues through mutational experiments and further structural analysis in the presence of co-factors, this information could be used to develop specific inhibitors of PgFold against this oral pathogen.

Author Contributions: Conceptualization, Y.-S.S. and J.H.L.; methodology, S.I., H.D. and J.H.; validation, Y.-S.S. and J.H.L.; formal analysis, S.I., H.D. and J.H.; investigation, S.I., H.D. and J.H.; resources, Y.-S.S. and J.H.L.; data curation, H.D.; writing—original draft preparation, S.I., H.D. and Y.-S.S.; writing—review and editing, Y.-S.S. and J.H.L.; funding acquisition, J.H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was a part of the project titled “Development of potential antibiotic compounds using polar organism resources (KIMST 20200610, KOPRI PM23030),” funded by the Ministry of Oceans and Fisheries, Republic of Korea.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank the staff at the X-ray core facility of the Korea Basic Science Institute (KBSI; Ochang, Republic of Korea) and BL-5C of the Pohang Accelerator Laboratory (Pohang, Republic of Korea) for their kind assistance with data collection.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rezk, B.M.; Haenen, G.R.M.M.; Van Der Vijgh, W.J.F.; Bast, A. Tetrahydrofolate and 5-methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore. *FEBS Lett.* **2003**, *555*, 601–605. [[CrossRef](#)] [[PubMed](#)]
2. Ames, T.D.; Rodionov, D.A.; Weinberg, Z.; Breaker, R.R. A eubacterial riboswitch class that senses the coenzyme tetrahydrofolate. *Chem. Biol.* **2010**, *17*, 681–685. [[CrossRef](#)] [[PubMed](#)]
3. Kim, S.J.; Yoon, J.; Im, D.K.; Kim, Y.H.; Oh, M.K. Adaptively evolved *Escherichia coli* for improved ability of formate utilization as a carbon source in sugar-free conditions. *Biotechnol. Biofuels* **2019**, *12*, 207. [[CrossRef](#)]
4. Burda, P.; Kuster, A.; Hjalmarson, O.; Suormala, T.; Bürer, C.; Lutz, S.; Roussey, G.; Christa, L.; Asin-Cayuela, J.; Kollberg, G.; et al. Characterization and review of MTHFD1 deficiency: Four new patients, cellular delineation and response to folic and folinic acid treatment. *J. Inherit. Metab. Dis.* **2015**, *38*, 863–872. [[CrossRef](#)] [[PubMed](#)]
5. Kim, S.E. Enzymes involved in folate metabolism and its implication for cancer treatment. *Nutr. Res. Pract.* **2020**, *14*, 95–101. [[CrossRef](#)] [[PubMed](#)]
6. Allaire, M.; Li, Y.; MacKenzie, R.E.; Cygler, M. The 3-D structure of a folate-dependent dehydrogenase/cyclohydrolase bifunctional enzyme at 1.5 Å resolution. *Structure* **1998**, *6*, 173–182. [[CrossRef](#)]

7. Murta, S.M.F.; Vickers, T.J.; Scott, D.A.; Beverley, S.M. Methylene tetrahydrofolate dehydrogenase/cyclohydrolase and the synthesis of 10-CHO-THF are essential in *Leishmania major*. *Mol. Microbiol.* **2009**, *71*, 1386–1401. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Eadsforth, T.C.; Gardiner, M.; Maluf, F.V.; McElroy, S.; James, D.; Frearson, J.; Gray, D.; Hunter, W.N. Assessment of *Pseudomonas aeruginosa* N⁵, N¹⁰—Methylenetetrahydrofolate dehydrogenase—Cyclohydrolase as a potential antibacterial drug target. *PLoS ONE* **2012**, *7*, e35973. [\[CrossRef\]](#) [\[PubMed\]](#)
9. De Berardinis, V.; Vallenet, D.; Castelli, V.; Besnard, M.; Pinet, A.; Cruaud, C.; Samair, S.; Lechaplais, C.; Gyapay, G.; Richez, C.; et al. A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol. Syst. Biol.* **2008**, *4*, 174. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Fu, C.; Sikandar, A.; Donner, J.; Zaburannyi, N.; Herrmann, J.; Reck, M.; Wagner-Döbler, I.; Koehnke, J.; Müller, R. The natural product carolacton inhibits folate-dependent C1 metabolism by targeting FolD/MTHFD. *Nat. Commun.* **2017**, *8*, 1529. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Shen, B.W.; Dyer, D.H.; Huang, J.Y.; D'Ari, L.; Rabinowitz, J.; Stoddard, B.L. The crystal structure of a bacterial, bifunctional 5, 10 methylene-tetrahydrofolate dehydrogenase/cyclohydrolase. *Protein Sci.* **1999**, *8*, 1342–1349. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Eadsforth, T.C.; Maluf, F.V.; Hunter, W.N. *Acinetobacter baumannii* FolD ligand complexes—Potent inhibitors of folate metabolism and a re-evaluation of the structure of LY374571. *FEBS J.* **2012**, *279*, 4350–4360. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Eadsforth, T.C.; Cameron, S.; Hunter, W.N. The crystal structure of *Leishmania major* N⁵, N¹⁰ -methylenetetrahydrofolate dehydrogenase/cyclohydrolase and assessment of a potential drug target. *Mol. Biochem. Parasitol.* **2012**, *181*, 178–185. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Bermingham, A.; Derrick, J.P. The folic acid biosynthesis pathway in bacteria: Evaluation of potential for antibacterial drug discovery. *BioEssays* **2002**, *24*, 637–648. [\[CrossRef\]](#)
15. Mysak, J.; Podzimek, S.; Sommerova, P.; Lyuya-Mi, Y.; Bartova, J.; Janatova, T.; Prochazkova, J.; Duskova, J. *Porphyromonas gingivalis*: Major periodontopathic Pathogen Overview. *J. Immunol. Res.* **2014**, *2014*, 476068. [\[CrossRef\]](#)
16. Marchesan, J.T.; Gerow, E.A.; Schaff, R.; Taut, A.D.; Shin, S.; Sugai, J.; Brand, D.; Burberry, A.; Jorns, J.; Lundy, S.K.; et al. *Porphyromonas gingivalis* oral infection exacerbates the development and severity of collagen-induced arthritis. *Arthritis Res. Ther.* **2013**, *15*, R186. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Aleksijević, L.H.; Aleksijević, M.; Škrlec, I.; Šram, M.; Šram, M.; Talapko, J. *Porphyromonas gingivalis* virulence factors and clinical significance in periodontal disease and coronary artery diseases. *Pathogens* **2022**, *11*, 1173. [\[CrossRef\]](#)
18. Nishihara, R.; Sugano, N.; Takano, M.; Shimada, T.; Tanaka, H.; Oka, S.; Ito, K. The effect of *Porphyromonas gingivalis* infection on cytokine levels in type 2 diabetic mice. *J. Periodont. Res.* **2009**, *44*, 305–310. [\[CrossRef\]](#)
19. Dominy, S.S.; Lynch, C.; Ermini, F.; Benedyk, M.; Marczyk, A.; Konradi, A.; Nguyen, M.; Haditsch, U.; Raha, D.; Griffin, C.; et al. *Porphyromonas gingivalis* in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Sci. Adv.* **2019**, *5*, eaau3333. [\[CrossRef\]](#)
20. Li, X.; Kolltveit, K.M.; Tronstad, L.; Olsen, I. Systemic diseases caused by oral infection. *Clin. Microbiol. Rev.* **2000**, *13*, 547–558. [\[CrossRef\]](#)
21. Kalhor, H.; Goodarzi, N.N.; Rahimi, H.; Firoozeh, F.; Badmasti, F. Identification of novel drug targets in *Porphyromonas gingivalis* and proposing inhibitors against acetate kinase using structure-based virtual screening. *Process Biochem.* **2023**, *130*, 310–321. [\[CrossRef\]](#)
22. Jepsen, K.; Falk, W.; Brune, F.; Fimmers, R.; Jepsen, S.; Bekereldjian-Ding, I. Prevalence and antibiotic susceptibility trends of periodontal pathogens in the subgingival microbiota of German periodontitis patients: A retrospective surveillance study. *J. Clin. Periodontol.* **2021**, *48*, 1216–1227. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Japoni, A.; Vazin, A.; Noushadi, S.; Kiany, F.; Japoni, S.; Alborzi, A. Antibacterial susceptibility patterns of *Porphyromonas gingivalis* isolated from chronic periodontitis patients. *Med. Oral Patol. Oral Cir. Bucal* **2011**, *16*, 1031–1035. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Conrads, G.; Klomp, T.; Deng, D.; Wenzler, J.S.; Braun, A.; Abdelbary, M.M. The Antimicrobial Susceptibility of *Porphyromonas gingivalis*: Genetic Repertoire, Global Phenotype, and Review of the Literature. *Antibiotics* **2021**, *10*, 1438. [\[CrossRef\]](#)
25. Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **1997**, *276*, 307–326. [\[CrossRef\]](#)
26. Winn, M.D.; Ballard, C.C.; Cowtan, K.D.; Dodson, E.J.; Emsley, P.; Evans, P.R.; Keegan, R.M.; Krissinel, E.B.; Leslie, A.G.; McCoy, A.; et al. Overview of the CCP4 suite and current developments. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67*, 235–242. [\[CrossRef\]](#)
27. Emsley, P.; Cowtan, K. Coot: Model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60*, 2126–2132. [\[CrossRef\]](#)
28. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Židek, A.; Potapenko, A.; et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583–589. [\[CrossRef\]](#)
29. Murshudov, G.N.; Skubák, P.; Lebedev, A.A.; Pannu, N.S.; Steiner, R.A.; Nicholls, R.A.; Winn, M.D.; Long, F.; Vagin, A.A. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67*, 355–367. [\[CrossRef\]](#)
30. Afonine, P.V.; Grosse-Kunstleve, R.W.; Echols, N.; Headd, J.J.; Moriarty, N.W.; Mustyakimov, M.; Terwilliger, T.C.; Urzhumtsev, A.; Zwart, P.H.; Adams, P.D. Towards automated crystallographic structure refinement with *phenix.refine*. *Acta Crystallogr. D* **2012**, *68*, 352–367. [\[CrossRef\]](#)

31. Chen, V.B.; Arendall, W.B., 3rd; Headd, J.J.; Keedy, D.A.; Immormino, R.M.; Kapral, G.J.; Murray, L.W.; Richardson, J.S.; Richardson, D.C. MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66*, 12–21. [[CrossRef](#)] [[PubMed](#)]
32. Williams, C.J.; Headd, J.J.; Moriarty, N.W.; Prisant, M.G.; Videau, L.L.; Deis, L.N.; Verma, V.; Keedy, D.A.; Hintze, B.J.; Chen, V.B.; et al. MolProbity: More and better reference data for improved all-atom structure validation. *Protein Sci.* **2018**, *27*, 293–315. [[CrossRef](#)] [[PubMed](#)]
33. Schrödinger, L.; DeLano, W. PyMOL. Version 2.0 Schrödinger, LLC. 2020. Available online: <http://www.pymol.org/pymol> (accessed on 10 October 2023).
34. Nelson, K.E.; Rleischmann, R.D.; DeBoy, R.T.; Paulsen, I.T.; Fouts, D.E.; Eisen, J.A.; Daugherty, S.C.; Dodson, R.J.; Durkin, A.S.; Gwinn, M.; et al. Complete genome sequence of the oral pathogenic bacterium *Porphyromonas gingivalis* Strain W83. *J. Bacteriol.* **2004**, *186*, 5591–5601. [[CrossRef](#)]
35. Matthews, B.W. Solvent content of protein crystals. *J. Mol. Biol.* **1968**, *33*, 491–497. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.