Published in final edited form as:

FEBS J. 2012 December ; 279(23): 4350–4360. doi:10.1111/febs.12025.

***Acinetobacter baumannii* FoD ligand complexes – potent inhibitors of folate metabolism and a re-evaluation of the structure of LY374571**

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Abstract

The bifunctional N^5, N^{10} -methylenetetrahydrofolate dehydrogenase/cyclohydrolase (DHCH or FoD), which is widely distributed in prokaryotes and eukaryotes, is involved in the biosynthesis of folate cofactors that are essential for growth and cellular development. The enzyme activities represent a potential antimicrobial drug target. We have characterized the kinetic properties of FoD from the Gram-negative pathogen *Acinetobacter baumannii* and determined high-resolution crystal structures of complexes with a cofactor and two potent inhibitors. The data reveal new details with respect to the molecular basis of catalysis and potent inhibition. A unexpected finding was that our crystallographic data revealed a different structure for LY374571 (an inhibitor studied as an antifolate) than that previously published. The implications of this observation are discussed.

Keywords

antifolate; cyclohydrolase; dehydrogenase; enzyme inhibition; X-ray structure

Introduction

Multidrug resistant bacteria represent a major health threat and an increasingly difficult problem in the hospital environment [1]. Drug-resistant Gram-negative bacteria such as *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, in particular, pose a significant therapeutic challenge with strains resistant to some or all common therapeutic agents [2-4]. New drugs are urgently sought to combat this problem, with different strategies being employed [5]. We have adopted a target-based approach [6] seeking to characterize, validate and assess specific enzymes for their potential to underpin early-stage drug antimicrobial discovery. Of particular interest is folate metabolism, which supplies the derivatives required to support essential biosynthetic pathways [7,8].

The enzymes of folate metabolism are key to the biosynthesis of thymidine, purines and amino acids (glycine and methionine), as well as the metabolism of histidine and serine [9,10]. Several enzymes modify tetrahydrofolate (THF) to supply methyl-, methylene, formyl- and unsubstituted THF derivatives. One important intermediate N^{10} -formylTHF can be formed by in two steps: from N^5, N^{10} -methyleneTHF to N^5, N^{10} -methenylTHF [by the

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Database: The coordinates have been deposited in the Protein Data Bank under accession numbers: [4B4U](#), [4B4V](#) and [4B4W](#).

NADP⁺ or NAD⁺ dependent N^5,N^{10} -methylenetetrahydrofolate dehydrogenase (DH)], which is subsequently hydrolyzed to N^{10} -formylTHF [by N^5,N^{10} -methenyltetrahydrofolate cyclohydrolase (CH)] (Fig. 1). These enzyme activities occur in different combinations. In bacteria, dehydrogenase and cyclohydrolase activities are provided by a bifunctional enzyme (annotated as FdD or DHCH). Many eukaryotes possess a longer poly-peptide, which includes an additional N^{10} -formylTHF ligase domain. A number of crystal structures have been reported and these include the cytosolic DC (dehydrogenase/cyclohydrolase) domain of the human trifunctional enzyme in complex with the cofactor and inhibitors [11,12], the enzyme from *Escherichia coli* [13] and *Leishmania major* [7]. In addition, the monofunctional NAD⁺ dependent yeast DH has been described [14]. We previously reported the structure and ligand discovery research targeting *P. aeruginosa* FdD (*Pa*FdD) [8]. However, we were unable to determine crystal structures of ligand complexes either with potent inhibitors or the novel hits that we identified. We sought an alternative and switched to the *A. baumannii* enzyme (*Ab*FdD) to provide structural data. In the present study, we describe the kinetic characterization and high-resolution crystal structures of *Ab*FdD, in the presence of ligands, substrate and catalytic intermediate mimics. The structures reveal molecular details relevant to the enzyme mechanism and serve to raise questions about previous proposals involving a channelling process. They inform on potent inhibition and, moreover, revealed an unexpected finding that forced a re-evaluation of a previously characterized FdD inhibitor: the antifolate LY374571.

Results and Discussion

Kinetic analysis

A highly efficient recombinant source of *Ab*FdD was established to supply material for characterization. The *Ab*FdD dehydrogenase activity was measured by monitoring the conversion of N^5,N^{10} -methylenetetrahydrofolate to N^5,N^{10} -methenyltetrahydrofolate. Measuring the decrease of N^5,N^{10} -methenyltetrahydrofolate provided the cyclohydrolase activity. The enzyme kinetic data are presented in Table 1. We purchased the substrate for the dehydrogenase assay, rather than producing it by condensation from tetrahydrofolate and formaldehyde. This aimed to avoid the additional reactivity of excess formaldehyde in the reaction mix. *Ab*FdD gave specific activities of 161.4 ± 5.7 and 350.2 ± 4.4 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ for the dehydrogenase and cyclohydrolase respectively, which, although high compared to the human DHCH (19 and 133 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) and *L. major* DHCH (22 and 6.3 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$), are comparable with values reported for bacterial enzymes. These include the *E. coli* and *Peptostreptococcus productus* enzymes, which showed a dehydrogenase activity of 200 and 627 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ respectively and that of *Clostridium formicoaceticum* with a cyclohydrolase activity of 469 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ [15-19]. The K_m values determined for NADP⁺, the N^5,N^{10} -methylenetetrahydrofolate substrate and N^5,N^{10} -methenyltetrahydrofolate intermediate, or second substrate, are 71.9 ± 6.0 , 26.8 ± 2.2 and 40.3 ± 6.9 μM respectively. The substrate analogue LY354899 has previously been shown to be a competitive inhibitor of the DH activity of human DHCH (29 nM), *Pa*FdD (30 nM) and *L. major* FdD (105 nM) [8,12,18]. However, little work has been carried out regarding cyclohydrolase activity, presumably with the assumption that inhibition against one activity will result in inhibition of the other. LY354899 and the compound designated as LY374571 gave K_i values with respect to the dehydrogenase activity of 116 ± 2.4 nM and 45.8 ± 4.1 nM, respectively. As will be explained, the structure of LY374571 is different from that of the compound characterized in the present study. Nevertheless, both compounds were significantly more potent inhibitors against the cyclohydrolase activity with K_i values of 7.1 ± 1.6 nM and 5.6 ± 1.7 nM respectively, suggesting that they represent good mimics of substrates, products or catalytic intermediates. These low K_i values molecules encouraged us to seek out structural data informing on the molecular basis of inhibition.

Overall structure

The crystal structure of the binary *Ab*Fold-NADP⁺ complex was solved to a resolution of 1.45 Å, whereas the ternary structures with the inhibitors were determined to a resolution of 2.0 Å (Table 2). In all three structures, the main chain atoms are well defined by the electron density, although there are several side chains in a flexible loop (residues 231–243) that were poorly ordered and assigned zero occupancy.

The *Ab*Fold subunit, a polypeptide of 282 amino acids, displays the tertiary structure typical of DHCH enzymes (Fig. 2). This consists of 11 α -helices and 11 β -strands forming a two-domain structure with the N-terminal domain encompassing residues 6–150 and the C-terminal domain encompassing residues 150–262. The N-terminal domain contains the catalytic site where the inhibitors bind and is discussed separately. The C-terminal domain displays the Rossmann fold and here the NADP⁺ cofactor binds in an extended conformation in a deep cleft. The amino acids that form the cofactor binding site are highly conserved [7,8]. In common with several other structures, in the present study, the *Ab*Fold cofactor complex shows well-defined electron density for the adenine and phosphate groups but not for the nicotinamide and associated ribose. The adenine binds in a hydrophobic pocket on the outer surface of the enzyme and, together with other components, participates in direct and solvent-mediated hydrogen-bond interactions to functional groups on the enzyme in a fashion similar to that described previously [7]. Structures of human DHCH [11] and *Thermoplasma acidophilum* FolD [17] show ordered NADP⁺ and superposition on the structure of *Ab*Fold highlights the conserved nature of the cofactor binding sites and also suggests how the nicotinamide binds adjacent to the catalytic centre (data not shown).

*Ab*Fold forms a dimer in solution, as revealed by size exclusion chromatography. This dimer, which is a common feature of FolD, constitutes the asymmetric unit of the crystal structures. The dimer interface is predominantly polar and primarily involves interactions formed between residues on α 5, α 7 and β 6 of noncrystallographically related subunits (Fig. S1). Approximately 10% (2880 Å²) of a subunit surface is occluded from solvent by dimer formation. The active site is distant from the dimer interface, suggesting that the dimerization is not necessary for activity. Superposition of the crystallographically independent subunits in the three structures gives rmsd values in the range 0.22–0.27 Å over 282 main chain atoms, indicating that the binary and ternary complexes display a highly similar overall structure. This value is significantly less than that seen for other DHCH enzymes, which show a significant difference between the binding clefts in the molecules in the asymmetric unit; this difference can be attributed to distinct domain orientations [7].

The structural basis of inhibition

The folate-binding site, the catalytic centre, is located in a deep, water filled pocket on the N-terminal domain formed principally by contributions from α 2, α 4, β 4 and α 10, as well as the linking regions between α 4– α 5, β 11– α 11 and the β 8– β 9 hairpin (Fig. 2). Although there are no gross conformational changes between the binary and ternary complexes, a specific difference is the placement of the Tyr49 side chain. In the presence of inhibitors, the side chain swivels to stack onto the *para*-aminobenzoate moieties. In addition, His98 adopts a dual conformation in the binary complex with one position occluding the active site but, in the presence of the inhibitors, this rotamer is not observed.

LY354899 binds in a bent conformation (Fig. 3A). The pteridine moiety is wedged between Gln97 and Ile169, forming direct hydrogen-bonding interactions to the side chain of Asp120, the main chain carbonyl of Leu96, the main chain carbonyl and amide of His98 and to the side chain of Lys53 (Fig. 3B). Solvent-mediated interactions also help link the pteridine O4 to the enzyme. As noted above, the side chain of Tyr49 forms π -stacking

interactions on one side of the *para*-aminobenzoate segment; on the other side, Gly262 and side chains of Thr265 and Ile266 (not shown) contribute to van der Waals interactions with the inhibitor. The glutamate tail of the inhibitor forms two direct hydrogen bonds with the enzyme. These involve Tyr49 OH and the amide of Gly239. The former helps to position the aromatic side chain over the *para*-aminobenzoate segment. Several solvent-mediated interactions (one of which is depicted in Fig. 3B) link the acidic tail to the enzyme. The side chains of Met52 (not shown) and Phe231, together with Pro238, form a hydrophobic clamp to assist positioning and binding of the tail.

Analysis of what we considered would be the LY374571 complex revealed an unexpected finding. Well-defined electron density indicated the presence of an inhibitory ligand. However, although there were similarities in respect of the *para*-aminobenzoate tail, the head group was obviously distinct and incompatible with the chemical structure assigned to LY374571 (Fig. 4A). By consideration of the hydrogen-bonding pattern, the synthetic route that was followed and analytical data, we assigned the inhibitor as 2-(4-(3-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)ureido) benzamido)pentanedioic acid. This compound is assigned the code PDB [9L9](#) in the Protein Data Bank (PDB).

The ligand binds to AbFold in a similar fashion to LY354899 exploiting functional groups on the enzyme in hydrogen bond formation and van der Waals interactions (Fig. 4B). The conservation of interactions can be explained by the close spatial agreement and hydrogen-bonding capacity of functional groups on the inhibitors. The similarity in the mode of binding to AbFold extends to the conservation of the water molecules that mediate links between the ligands and the enzyme. A chemical difference between the two inhibitors occurs at the link between heterocyclic moieties and the *para*-aminobenzoate segment. The ureido link provides two amide groups and they combine to form hydrogen bonds to a water molecule and this in turn participates in a solvent network in the active site. A minor difference involves the lack of a hydrogen bond between the glutamate tail and Tyr49 OH in the case of the ureido compound. Here, the tail adopts a slightly different orientation, with one carboxylate group directed out of the active site.

We had followed the reported synthetic route to LY374571 and our analytical data were in excellent agreement with that reported previously [12]. This is to be expected given that the compounds are structural isomers. The highly-defined electron density in the AbFold crystal structure, together with the sensible chemical interactions, in particular the hydrogen-bonding assignments, provides confidence in our structure. The synthetic method may have produced a different compound in our hands or the original assignment of the LY374571 structure was in error. We sought to understand the discrepancy and so considered the evidence for the LY374571 structure and also the factors involved in the synthetic reaction.

The study describing the complex of human DHCH with LY374571 [12] did not provide an illustration of the electron density for the ligand; therefore, we calculated an omit difference density map using the deposited structure factors (Fig. S2). The map shows a density that might represent water molecules but certainly not a bound, well-ordered inhibitor. The occupancy of the LY374571 atoms had been set at 0.25 in the complex to obtain *B*-factors comparable to that observed for active site residues. This approach might not be considered best practice, potentially leading to phase bias and misinterpretation. The structures described in the present study show an unambiguous density for both ligands, which have been refined with full occupancy and display *B*-factors that are significantly less than the overall *B*-factor of the protein atoms (Table 2). The DHCH-LY374571 structure is also deficient in terms of chemical sense. The pyrimidine ring is positioned differently compared to our structure (Fig. 5) and the six associated functional groups make only a single hydrogen bond to the protein and few van der Waals contacts. For a compound that

displays such a low K_i , this might be considered unusual. In addition, the conformation of LY374571 places two hydrogen bond donor groups, NH and NH₂, approximately 2.2 Å apart; this is implausible. By contrast in our complex, five of the functional groups form direct hydrogen bonds in addition to extensive van der Waals interactions with residues in *AbFolD* that are highly conserved in orthologues and there are no intramolecular steric clashes.

A key step in the synthetic procedure is the reaction of 6-hydroxy-2,4,5-triaminopyrimidine with ethyl 4-iso-cyanatobenzoate. To make the compound that we assigned, the coupling would have occurred at the amine (N5) adjacent to the pyridone oxygen. Of the three amines on the starting material, this might be predicted to be the most nucleophilic. The least nucleophilic, and the position where a reaction to produce LY374571 is required, is probably at N4. We therefore conclude there is a strong possibility that the compound previously assigned as LY374571 is the compound we have characterized in the present study.

As a result of synthesizing the ‘wrong’ compound, we next attempted (several times) to produce LY374571 using different approaches, principally by changing the starting material in an attempt to achieve selective addition onto the ethyl-4 isocyanatobenzoate. On each occasion, the synthesis failed or produced mixtures, with the predominant species being the compound described. Further synthetic protocols would have to be tried to obtain the ‘real’ LY374571.

Reaction mechanism

The inhibitors that we have characterized represent good mimics for the intermediate, N^{δ},N^{10} -methenylTHF, and the reaction product, N^{10} -formylTHF, and allow us to comment on aspects of the enzyme action. The enzyme *FolD* possesses both the Rossmann-fold and the YxxxK motif typical of the small dehydrogenase/reductase (SDR) family. In the SDR family, the lysine interacts with the ribose and serves to position the nicotinamide, whereas the tyrosine provides a hydroxyl group to participate directly in catalysis [20]. In *AbFolD*, the residues in the motif are Tyr49 and Lys53 but, because the substrate is placed between them and the cofactor, preventing any direct interaction, then their contribution to the mechanism is distinct from that in the SDR family. The lysine, which is in a mainly hydrophobic environment as a result of the positions of Ile266, Val250, Leu269, Ile270, Ile95, Ala56, Tyr49 and Leu35 (not shown) likely acts in conjunction with Gln97 to provide the right environment to position and activate substrate, and to support hydride abstraction by NADP⁺ for the dehydrogenase activity. Although, in our structures, the nicotinamide is disordered and absent from the model, a superposition of human DHCH (PDB code: [1DIB](#)) [12] with an ordered cofactor on *AbFolD* ligand complexes placed the reactive C4 group within 3 Å of the position where hydride abstraction occurs, therefore implying that the dehydrogenase reaction is a relatively simple process. This is consistent with previous studies [12].

However, there is speculation [12,21,22] that, for a bifunctional process to occur, the pteridine had to change orientation after hydride abstraction. It was suggested that a channelling process was necessary to support the cyclohydrolase reaction. The structural data reported in the present study (Fig. 3B) suggest that this may not be required. The complex with LY354899 in particular reveals ordered water molecules binding near to where hydrolysis occurs. Several functional groups (e.g. provided by Lys53, Asp120 and Thr265) are available to contribute by activating water to participate in the second cyclohydrolase reaction. Less likely but still worthy of mention is the possibility that Tyr49 might undergo a conformational change, similar to that observed in going from binary to

ternary complex, and provide a hydroxyl group to help activate a water in support of the cyclohydrolase reaction.

Concluding remarks

Structures of *AbFolD* have been determined in the presence of partially disordered NADP⁺ and two potent inhibitors, both of which are reaction intermediate mimics. The structures clearly resolve the details of molecular recognition of these inhibitors with the target enzyme. These structural data contribute new information on the reaction mechanism and support the assignment of an alternative structure for a previously studied FolD inhibitor, LY374571.

Experimental procedures

Recombinant protein production

The *A. baumannii folD* gene, encoding the bifunctional DHCH, was identified in UniProtKB (<http://www.uniprot.org> accession number [D0CBC8](#)). The gene (locus tag: EEX03016.1) was amplified from genomic DNA (American Type Culture Collection strain 19606) with primers carrying *Xho*I and *Bam*HI restriction sites (bold), respectively: 5'-CTC-GAG-ATG-GCA-TTG-GTT-TTA-GA-3', 5'-GGA-TCC-TTA-ACC-TAA-AGC-TTT-TTT-TTC-AG-3'. The PCR product was ligated into pCR-BluntII-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA) before excising the gene and subsequent ligation into a modified pET15b vector (Novagen, Madison, WI, USA). This vector contains a tobacco etch virus (TEV) protease recognition sequence in place of thrombin (pET15bTEV), resulting in the product carrying an N-terminal hexahistidine tag (His-tag), which is cleavable with TEV protease. The recombinant plasmid was amplified in XL-1 blue *E. coli*, then heat-shock transformed into *E. coli* BL21 (DE3) for protein production. The integrity of the gene sequence was verified by the University of Dundee Sequencing Service.

E. coli harbouring the expression plasmid were cultured at 37 °C, with shaking at 200 rpm, in auto-induction media [23] supplemented with 50 mg·L⁻¹ carbenicillin for approximately 3 h until *D*₆₀₀ of 0.8 was reached. The temperature was subsequently reduced to 21 °C for a further 22 h, before the cells were harvested by centrifugation (3500 g for 30 min at 4 °C). The cells were resuspended into a lysis buffer (buffer A: 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM imidazole) containing DNase I (200 µg) and an EDTA-free protease-inhibitor cocktail tablet (Roche, Basel, Switzerland). Cells were lysed using a French press at 16 000 Ψ and the solution clarified by centrifugation (50 000 g for 30 min at 4 °C). The supernatant was filtered, loaded onto a HisTrap HP 5-mL column (GE Healthcare, Milwaukee, WI, USA) pre-charged with Ni²⁺ and the His-tagged *AbFolD* eluted using a imidazole gradient of 25 mM to 1 M. The fractions were analyzed using SDS/PAGE and those found to contain *AbFolD* were pooled. His-tagged TEV protease (1 mg per 20 mg of *FolD*) was added and the mixture dialyzed against 50 mM Tris-HCl (pH 7.5) and 250 mM NaCl for 3 h at room temperature. Another passage through a His-Trap column separated cleaved *AbFolD* from TEV protease, the cleaved His-tag peptide and uncleaved His-tagged *AbFolD*. The sample of *AbFolD* was then applied to a Superdex 200 26/60 column (GE Healthcare), pre-equilibrated in buffer A minus imidazole. The protein eluted as a single species with a mass of approximately 60 kDa corresponding to a dimer. The sample was dialysed into 100 mM Pipes (pH 7.0) and 150 mM NaCl and concentrated to 20 mg⁻¹·mL⁻¹ using a 10-kDa MWCO Amicon Ultra devices (Millipore, Billerica, MA, USA). Protein concentration was determined spectrophotometrically using a theoretical extinction coefficient of 10 220 M⁻¹·L⁻¹·cm⁻¹ at 280 nm calculated using PROTPARAM [24]. The high level of protein purity was confirmed by SDS/PAGE and MALDI-TOF MS (FingerPrint Proteomic Facility, University

of Dundee). The yield of purified protein was approximately $60 \text{ mg}^{-1} \cdot \text{L}^{-1}$ of bacterial culture. Protein was diluted to $10 \text{ mg}^{-1} \cdot \text{mL}^{-1}$ and used fresh for crystallization trials or to $2 \text{ mg}^{-1} \cdot \text{mL}^{-1}$ and supplemented with 10% glycerol and then flash frozen in liquid nitrogen for storage before kinetic characterization.

Crystallization

Crystallization conditions were screened with the sitting drop vapour diffusion method in 96-well plates using a Phoenix liquid handling system (Rigaku; Art Robins Instruments, Mountain View, CA, USA) and commercially available screens. Optimized conditions were achieved using hanging drop vapour diffusion methods with drops consisting of $2 \mu\text{L}$ of protein solution and $2 \mu\text{L}$ of reservoir solution. Crystals of the *AbFolD* were obtained using a mixture of $2 \mu\text{L}$ of reservoir solution (0.1 M Bis-Tris, pH 5.5, 25% poly(ethylene glycol) 3350, 0.2 M MgCl_2 and 2% dioxane) and with an equal volume of protein solution (*AbFolD* at $10 \text{ mg}^{-1} \cdot \text{mL}^{-1}$ and 10 mM NADP^+). Monoclinic crystals ($350 \times 200 \times 50 \mu\text{m}$), grew after approximately 5 days at room temperature. Crystals of the ternary complexes with the poorly soluble inhibitors were obtained after incubating the protein solution for 4 h with solid compounds in vast molar excess, before clarification by centrifugation and then using the supernatant for crystallization. Crystals of the *AbFolD* ternary complexes were obtained using a mixture of $0.75 \mu\text{L}$ of reservoir solution [0.1 M Bis-Tris, pH 5.5, 20% poly(ethylene glycol) 3350, 0.2 M MgCl_2 and 2% dioxane] and $1.5 \mu\text{L}$ volume of protein solution (*AbFolD* at $5 \text{ mg}^{-1} \cdot \text{mL}^{-1}$ and 10 mM NADP^+). The crystals were slightly smaller ($200 \times 100 \times 100 \mu\text{m}$). Crystals were transferred to a solution containing the reservoir solution adjusted to incorporate 20% glycerol before flash freezing at -173°C .

Data collection and structure solution

Data for the ternary complex crystals were collected in-house with a Micromax 007 rotating anode generator ($\lambda = 1.5418 \text{ \AA}$) and R-AXIS IV⁺⁺ dual image plate detector (Rigaku). Data for the binary complex were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) at beam line ID29 ($\lambda = 0.981 \text{ \AA}$) using an ADSC Q315r charge-coupled device detector. Processing and scaling of the data were carried out using *MOSFLM* [25] and *SCALA* [26]. The crystals are isomorphous (Table 2). There are two subunits in the asymmetric unit with V_m values of $2.44 \text{ \AA}^3 \cdot \text{Da}^{-1}$ and a bulk solvent content of approximately 50%. The initial NADP^+ binary complex structure was solved by molecular replacement using *PHASER* [27]. A subunit of the *E. coli* apo structure (PDB code: 1B0A) [13] provided the search model after it was changed to poly-alanine in *CHAINSAP* [28]. Refinement of the ternary complexes was initiated using the binary complex model. Refinement was carried out using *REFMAC5* [29] interspersed with electron-density and difference density map inspection, model manipulation and the incorporation of solvent and ligands using *COOT* [30]. The two monomers in the asymmetric unit were treated independently during refinement. translation/liberation/screw analysis [31] was applied to the ternary complex structures but not the binary complex, where anisotropic refinement of thermal parameters was carried out as a result of the high resolution of the available data. Crystallographic statistics are provided in Table 2. Model geometry was analyzed using *MOLPROBITY* [32], whereas rmsd values comparing structures were obtained from *LSQKAB* [33]. Figures were prepared with *PYMO* [34] and *CHEMDRAW* (CambridgeSoft, Cambridge, MA, USA). Amino acid sequence alignments were carried out using *MUSCLE* [35] and visualized in *ALINE* [36].

Kinetic characterization

The substrates (*6R,S*)-5,10-methylene-5,6,7,8-tetrahydrofolic acid and (*6R,S*)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid were purchased from Schircks Laboratories (Jona, Switzerland). Two compounds were identified as potential inhibitors of *AbFolD*: 5,6,7,8-tetrahydro-*N*⁵,*N*¹⁰-carbonylfolic acid and (*2R*)-2-[(4-[(2,5-diamino-6-

hydroxypyrimidinyl)carbamoyl]amino}phenyl)formamido] pentanedioic acid. These compounds, previously developed by Lilly and assigned the names LY354899 and LY374571, respectively, were synthesized in accordance with reported methods [12,37,38] and analyzed by NMR, MS and HPLC (Figs S3-S6, Doc. S1). All of the chemicals utilized were of analytical grade.

The enzyme was assayed using a protocol reported previously [39] with modifications. K_m values for the dehydrogenase activity with N^5,N^{10} -methylene tetrahydrofolate were determined at 27 °C in 25 mM Mops (pH 7.3), 30 mM 2-mercaptoethanol, using between 2.5 μ M and 1 mM substrate (dissolved in 20 mM NaOH). The reaction was initiated with addition of 1 mM NADP⁺, whereas assays for the cofactor were fixed at 250 μ M N^5,N^{10} -methylene tetrahydrofolate and between 2.5 μ M and 1 mM NADP⁺. Assays were run in duplicate in 0.5-mL volumes in acrylic cuvettes and were stopped with an equal volume of 1 M HCl after 2 min. Background rates were determined with pre-stopped reactions. Concentrations of intermediate were determined using the extinction co-efficient of 24.9 mM⁻¹.cm⁻¹ at 350 μ m for acidified solutions of the intermediate [40]. Unlike previously described inhibition studies [12,18], the substrate was used directly rather than being formed from tetrahydrofolate and formaldehyde during the reaction.

The assays for cyclohydrolase activity with N^5,N^{10} -methenyl tetrahydrofolate were performed at 27 °C in 25 mM Mops (pH 7.3), 30 mM 2-mercaptoethanol and between 0.5 and 100 μ M substrate (dissolved in 10 mM HCl) and started by the addition of the enzyme. Assays were in duplicate in 1-mL volumes in acrylic cuvettes with the hydrolysis of N^5,N^{10} methenyl tetrahydrofolate measured as a decrease at 355 nm and a background rate determined in enzyme free assays. Concentrations of the substrate were calculated using the extinction coefficient of 25.1 mM⁻¹.cm⁻¹ [39]. Inhibition studies were carried out at the substrate K_m using at least ten concentrations of the inhibitors (dissolved in 100% dimethylsulfoxide) with the final dimethylsulfoxide concentrations in the assays never exceeding 1%. Activities for the inhibition assays are expressed as the percentage of a dimethylsulfoxide control. Absorbance for both assays was measured using a Shimadzu UV-2450 (Shimadzu Corp., Kyoto, Japan) and data were plotted using SIGMAPLOT, version 11 (Systat Software Inc., Chicago, IL, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Natalia Shpiro for the custom synthesis of inhibitors, Colin Gibson for synthetic chemistry input, David Norman for discussions on NMR, our colleagues in the Aeropath project and staff at the ESRF for excellent support. This work was funded by the Wellcome Trust (grants 082596 and 083481) and the European Commission Seventh Framework Programme (FP7/2007-2013; Aeropath).

Abbreviations

CH	N^5,N^{10} -methenyltetrahydrofolate cyclohydrolase
DH	N^5,N^{10} -methylenetetrahydrofolate dehydrogenase
DHCH	N^5,N^{10} methylenetetrahydrofolate dehydrogenase/cyclohydrolase
PDB	Protein Data Bank
SDR	small dehydrogenase/reductase

TEV	tobacco etch virus
THF	tetrahydrofolate.

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Fig. 1.

The enzymatic pathway of *AbFolD* showing (left) the chemical structure of tetrahydrofolate as a reference. (right) The two stages of the reaction catalysed by *AbFolD*, starting with the NADP⁺ reduction of *N*⁵,*N*¹⁰ methylene tetrahydrofolate to *N*⁵,*N*¹⁰ methenyl tetrahydrofolate, followed by the subsequent hydrolytic opening of the ring to form *N*¹⁰ formyl tetrahydrofolate.

Fig. 2. Ribbon diagram of an AbFolD subunit. An inhibitor (green sticks) and NADP⁺ (yellow sticks) occupy the catalytic and cofactor binding sites, respectively. α -helices and β -strands are labelled and coloured purple and blue, respectively.

Fig. 3. LY354899 bound to *AbFolD*. (A) The $F_o - F_c$ omit map (blue chicken wire) contoured at 2σ and the chemical structure of LY354899. (B) Details of binding in the active site. Residues and waters (blue spheres) that interact are labelled. Dashed bonds represent potential hydrogen-bonding associations. The fragment of the cofactor that could be modelled is shown as sticks with atomic positions coloured yellow for C, blue for N, orange for P and red for O. The C atoms of LY354899 and the *FolD* residues are green and grey, respectively.

Fig. 4.

A new inhibitor bound to *Abf1D*. The inhibitor is 2-(4-(3-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)ureido)benzamido)pentanedioic acid and has been assigned the code **9L9** in the PDB. A similar colour scheme as in Fig. 3 is applied, except the C atoms of the inhibitor are coloured cyan. (A) Left: the $F_o - F_c$ omit map contoured at the 2σ level. Centre: the chemical structure assigned of **9L9**. Right: the chemical structure assigned to LY374571. (B) Residues and waters that interact with the new pyrimidine derivative.

Fig. 5. Superposition of LY354899 (blue), 9L9 (green) from the *AbFold* complex crystal structures and the structure assigned as LY374571 (black) bound in the human DHCH structure.

Table 1Kinetic parameters for *Ab*Fold.

	K_m	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
First substrate	26.8 ± 2.2	88.3 ± 2.0	3.2×10^6
NADP+	71.9 ± 6.0	84.7 ± 2.0	1.1×10^6
Second substrate	40.3 ± 6.9	141.6 ± 11.3	3.5×10^6

Table 2

Data collection and refinement statistics.

	NADP ⁺ complex	NADP ⁺ -inhibitor complex	NADP ⁺ -LY354899 complex
Spacegroup	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Unit cell parameters, <i>a</i> , <i>b</i> , <i>c</i> , (Å) and β	55.0, 80.1, 68.6, 107°	55.5, 80.9, 68.5, 107°	55.2, 80.5, 68.5, 107°
Resolution range (Å) ^a	52.6–1.45 (1.53–1.45)	20.86–2.00 (2.11–2.00)	20.67–2.00 (2.11–2.00)
Number of measurements	357 135 (47 440)	91 096 (12 233)	87 114 (11 109)
Number of unique reflections	100 394 (14559)	38 850 (5634)	36 783 (4948)
Multiplicity	3.6 (3.3)	2.3 (2.2)	2.4 (2.2)
Completeness (%)	99.7 (99.3)	99.0 (99.1)	95.2 (88.4)
Mean <i>I</i> /σ <i>I</i>	13.1 (2.9)	18.7 (8.1)	12.9 (6.3)
Wilson <i>B</i> (Å ²)	14.9	18.9	15.9
<i>R</i> _{merge} ^b	0.055 (0.423)	0.030 (0.095)	0.044(0.118)
<i>R</i> _{work} ^c / <i>R</i> _{free} ^d	0.122/0.152	0.171/0.214	0.168/0.208
rmsd bonds (Å)	0.0085	0.0051	0.005
rmsd angles (°)	1.478	1.146	1.132
Ramachandran plot (%)			
Favoured/allowed	99.0/1.0	98.1/1.9	98.2/1.8
Protein residues/atoms	568/4329	564/4175	564/4207
Overall <i>B</i> (Å ²) chain A/B	16.9/16.4	22.1/19.1	16.7/15.2
Waters	740	592	654
Overall <i>B</i> (Å ²)	30.5	24.8	21.2
NADP ⁺	2	2	2
Overall <i>B</i> (Å ²)	27.2/26.1	27.7/25.2	24.2/23.3
Inhibitors	–	2	2
Overall <i>B</i> (Å ²)	–	18.0/15.8	12.9/12.1
Poly(ethylene glycol)/glycerol/Cl [–]	1/5/5	–/2/1	–/2/1
Overall <i>B</i> (Å ²)	39.2/32.2/20.7	30.8/17.4	30.9/14.6

^aValues in parentheses refer to the highest resolution bin.^b $R_{\text{merge}} = \frac{\sum h \sum i |I(h,i) - \langle I(h) \rangle|}{\sum h \sum i I(h,i)}$.^c $R_{\text{work}} = \frac{\sum h k l ||F_{\text{O}}| - |F_{\text{C}}||}{\sum |F_{\text{O}}|}$, where *F*_O is the observed structure factor and *F*_C is the calculated structure factor.^d*R*_{free} is the same as *R*_{work}, except it is calculated using 5% of the data that are not included in any refinement.