Mechanism of N¹⁰-formyltetrahydrofolate synthetase derived from complexes with intermediates and inhibitors

Lesia R. Celeste,¹ Geging Chai,¹ Magdalena Bielak,¹ Wladek Minor,²
Leslie L. Lovelace,¹ and Lukasz Lebioda^{1,3*}

¹Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208

²Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908

³Center for Colon Cancer Research, University of South Carolina, Columbia, South Carolina 29208

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Abstract: N¹⁰-formyltetrahydrofolate synthetase (FTHFS) is a folate enzyme that catalyzes the formylation of tetrahydrofolate (THF) in an ATP dependent manner. Structures of FTHFS from the thermophilic homoacetogen, *Moorella thermoacetica*, complexed with (1) a catalytic intermediate—formylphosphate (XPO) and product—ADP; (2) with an inhibitory substrate analog—folate; (3) with XPO and an inhibitory THF analog, ZD9331, were used to analyze the enzyme mechanism. Nucleophilic attack of the formate ion on the gamma phosphate of ATP leads to the formation of XPO and the first product ADP. A channel that leads to the putative formate binding pocket allows for the binding of ATP and formate in random order. Formate binding is due to interactions with the gamma-phosphate moiety of ATP and additionally to two hydrogen bonds from the backbone nitrogen of Ala276 and the side chain of Arg97. Upon ADP dissociation, XPO reorients and moves to the position previously occupied by the beta-phosphate of ATP. Conformational changes that occur due to the XPO presence apparently allow for the recruitment of the third substrate, THF, with its pterin moiety positioned between Phe384 and Trp412. This position overlaps with that of the bound nucleoside, which is consistent with a catalytic mechanism hypothesis that FTHFS works via a sequential ping-pong mechanism. More specifically, a random bi uni uni bi ping-pong ter ter mechanism is proposed. Additionally, the native structure originally reported at a 2.5 Å resolution was redetermined at a 2.2 Å resolution.

Keywords: formyltetrahydrofolate synthetase; folate enzymes; ping-pong mechanism; formylphosphate; ligase

Introduction

N¹⁰-Formyltetrahydrofolate synthetase (FTHFS; EC 6.3.4.3) catalyzes the formylation of tetrahydrofolate (THF) in an ATP-dependent manner shown in Reaction 1.

Abbreviations: APUC, asymmetric part of the unit cell; DTT, dithiothreitol; fTHF, formyltetrahydrofolate; FTHFS, N¹⁰-formyltetrahydrofolate synthetase; KMB, potassium maleate buffer; PABA, para-aminobenzoic acid; PDB, protein data bank; PEG, polyethylene glycol; THF, tetrahydrofolate; XPO, formylphosphate.

Additional Supporting Information may be found in the online version of this article.

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*Correspondence to: Lukasz Lebioda, Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter St., Columbia, SC 29208. E-mail: lebioda@mail.chem.sc.edu

This reaction occurs via a two-step mechanism in which the production of a formylphosphate (XPO) intermediate is followed by formation of the product, N¹⁰-formyltetrahydrofolate (fTHF). In prokaryotes, Reaction 1 is used in the biosynthesis of purines and also of Met-tRNA^{Met} and is followed by dehydration and subsequent reduction, creating one-carbon donors required for biosynthesis of pyrimidines and amino acids.¹ FTHFS is involved in C₁ carbon fixation processes for cellular biosynthesis and has an increased concentration in bacteria that perform both acetogenesis, from the Wood-Ljungdahl pathway of autotrophic CO₂ fixation, and purinolysis, through the glycine synthase/reductase pathway.^{1–4}

In contrast to bacterial FTHFS, the eukaryotic enzyme is more complex and is referred to as C₁-THF synthase. This enzyme is trifunctional and contains not only the activity of FTHFS, but also N^{5,10}-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) and N^{5,10}-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) activities.^{1,5} Presumably, its biochemical role is to keep formate and formaldehyde in chemically balanced states: available for biochemical processes, but not in excess as to pose a threat to the cell. Interestingly, the polymorphism of this gene is associated with the risk for migraine.⁶

FTHFS has been purified to homogeneity from several *Clostridium* species including: *thermoacetica* (now reclassified as *Moorella thermoacetica*), *acidurici*, and *cylindrosporum*. Comparison of the FTHFS sequence from these species shows a highly conserved sequence, which leads to a high degree of similarity in enzymatic, chemical, and physical properties.⁷

The enzyme is composed of four identical subunits each with a molecular mass of about 60,000 Da.^{1,8} Each subunit is composed of three domains: one larger and two smaller, which are organized around three mixed β-sheets.⁷ Subunit association of the tetrameric structure occurs through dimeric intermediates as was indicated through electron microscopy.⁹ The dimer-forming contacts differ from those involved in tetramer formation and the molecule is “X” shaped and relatively flat (Supporting Information Fig. S1). FTHFS requires monovalent cations for maximal catalytic activity as well as for maximal thermal stability.^{10,11}

Numerous experiments have been performed to determine the mechanism by which FTHFS operates. It has been suggested that FTHFS employs a random sequential mechanism (random ter ter mechanism)^{12–14} on the basis of steady-state kinetic data¹² and isotope exchange experiments.^{8,15} However, a more recent, careful kinetic study of FTHFS reports inhibition of the reaction by higher concentrations of THF.⁹ This phenomenon is not consistent with the random sequential mechanism.

Additionally, the participation of a non-dissociable, strongly bound formylphosphate intermediate (XPO) was postulated.^{16,17} Studies showed that chemically synthesized XPO can participate in both the forward and reverse reactions by formylating THF or phosphorylating ADP, respectively.^{18,19} The finding that ¹⁸O is transferred from formate to inorganic phosphate during the forward reaction¹⁵ further supports the involvement of this intermediate.

ZD9331 (Structure I), a novel antifolate developed by AstraZenecaTM, is under consideration for the treatment of several cancer forms.

Although ZD9331 was specifically developed to inhibit thymidylate synthase (TS) activity, it has also been shown to be an inhibitor of FTHFS activity.⁹ Another inhibitor of FTHFS activity is folate, the four electron oxidized version of THF, which was thought to bind within the active site in a similar manner to THF.²⁰

In this study, we report the outcome of partial FTHFS reactions as monitored by crystallography. These results confirm the formation of XPO when ATP reacts with formate, in the absence of THF. The

reaction yielded an FTHFS complex with ADP and XPO, which crystallized. In the presence of ATP, formate, and ZD9331, the reaction was carried out in crystals and proceeded with the production of XPO and a complex with this intermediate and ZD9331 was formed. The observed large overlap of the nucleoside binding site and the folates binding site is inconsistent with the random ter ter mechanism because the huge steric hindrance would prevent simultaneous binding of ATP and THF. These results lead to the ping-pong mechanism shown in Figure 1 and are entirely consistent with observed FTHFS inhibition by high concentrations of THF.⁹ Additionally, the structure of an inhibitory complex of FTHFS with the folate ion shows non-productive mode of binding and explains some of the previous data based on which the ter ter mechanism was proposed.

Results

Overall structure of FTHFS

The structures reported here are in agreement with those determined previously. [Atomic coordinates and structure factors of N¹⁰-formyltetrahydrofolate synthetase have been deposited in the Protein Data Bank under PDB IDs (3PZX) native, (3QB6) folate complex, (3RBO) ADP + formylphosphate complex, and (3SIN) ZD9331 + formylphosphate complex.] Out of 559 they include residues 5–409 and 412–558, with a two amino acid deletion of residues 410 and 411. Clear electron density can be seen for Ser409, followed directly by Trp412. The eliminated residues, Glu410 and Val411, are present in all other sequences in the NCBI database and were deleted in the clone used for these studies. This deletion however does not have an affect on the catalytic properties of the enzyme.⁷ The complexes of FTHFS-folate and FTHFS-ZD9331-XPO are very similar to that of the native enzyme. Only significant differences observed between these structures are found within the active sites and relate to ligand binding (Supporting Information Fig. S2A–S2C). In contrast, the complex of FTHFS-ADP-XPO shows significant differences from that of the native enzyme and the other complexes. These differences may be due to the crystals being non-isomorphous and thus the molecules having different crystal lattice contacts, or due to ADP/XPO binding in both subunits (Supporting Information Fig. S3A–S3B).

Structure of native FTHFS

Previously deposited structures of FTHFS only included residues 7–409 and 412–557, the structures reported here have clear density for additional residues at both the C and N termini. Electron density quality for a large portion of subunit B is distinctly

Figure 1. Schematic of the random bi (two substrates, formate and ATP, bind independently of one another), uni (first product, ADP, is released), uni (a third substrate, THF binds), bi (release of two products, phosphate and N¹⁰-formyltetrahydrofolate in random order) ping-pong ter ter FTHFS mechanism.

lower than that of the corresponding part of subunit A. The density for a part of subunit A is shown in Supporting Information Figure S4. Accordingly, the refined temperature factors are higher in subunit B. A superposition of the previously determined, at 2.5 Å resolution, structure of native FTHFS (PDB code 1EG7) with the structure reported here, refined at 2.2 Å resolution, yielded a rmsd. between the positions of C α of 0.49 Å, with the largest deviation in subunit A of 5.6 Å and 2.6 Å in subunit B.

Structure of FTHFS complex with ADP and XPO

The presence of the substrates in crystallization medium led to non-isomorphous crystals. This structure, refined at 2.5 Å resolution, showed ligands bound in both subunits. The electron density within the active site pockets is consistent with the products of the first step in the reaction: XPO and ADP (Fig. 2). The adenine of ADP is sandwiched between Phe384 and Trp412. The diphosphate of ADP is the acceptor of hydrogen bonds from the peptides of Gly73, Lys74, Thr75, and Thr76 and the side chain of Lys74. The XPO intermediate, for which the electron density is clearly separated from that of ADP, is also stabilized through hydrogen bonding with the side chains of Arg97 and Lys74 and main chain nitrogen atoms of Ala276 and Phe304.

The tight fit of the adenine moiety to its binding pocket is shown in Supporting Information Figures S5A–S5D. However, a channel leading to the putative formate ion binding site should allow this substrate to bind even when the ATP ion is present in the active site.

Structure of FTHFS complex with folate

The structure of FTHFS in complex with the folate molecule was determined at 3.0 Å resolution. Electron density reveals that the substrate analog is only bound in subunit A and its occupancy is 0.75 (Supporting Information Fig. S6). The pterin moiety of the folate is

Figure 2. The active site in the FTHFS-ADP-XPO complex. A: Stereoview of the F_o-F_c electron density (shown at 2σ level) corresponding to ADP and XPO in the FTHFS active site in subunit. B: XPO in the FTHFS active site in subunit B with corresponding hydrogen bond distances. The composite omit electron density map is shown in blue (contoured at 0.8σ level) and the F_o-F_c electron density map is shown in green (contoured at 2σ level). An interactive view is available in the electronic version of the article. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bound between the side chains of Trp412 and Phe384, partially overlapping with the nucleotide binding site. The para-aminobenzoic acid (PABA) and the glutamate group of the folate, however, are not positioned within the active site pocket, but are pointing in the opposite direction, indicating a non-productive mode of binding. PABA forms hydrophobic contacts with Pro385 and Leu408, whereas the glutamate group forms a hydrogen bond with the hydroxyl of Tyr396.

Structure of FTHFS complex with ZD9331 and XPO

This structure was determined at 2.67 Å resolution. Similar to the folate complex, the ZD9331 substrate analog is bound only in subunit A. The two fused rings (the pterin-like part) of ZD9331 have strong electron density, while the rest of ZD9331 is disordered. The pterin-like moiety of ZD9331 is bound

between the side chains of Trp412 and Phe384, partially overlapping with the nucleoside binding site, as also seen in the folate complex (Fig. 3). Contrary to the non-productive binding of the folate ion, density for ZD9331 suggests a mode of binding that is likely analogous to that of the substrate. A positive density feature in the difference map is seen at residues 71–76; it appears likely this density represents the bound XPO generated by the enzyme from the substrates present in the crystallization medium (Fig. 4). The position of XPO is shifted from that observed in the FTHFS-ADP-XPO complex by 5.5 Å, it also is rotated, which exposes its C-atom to nucleophilic attack by N¹⁰ of THF. None of the residues binding XPO in the FTHFS-ADP-XPO complex forms a contact in the new position (Supporting Information Fig. S7). There is a density for a sulfate ion close to the XPO binding site seen in the FTHFS-ADP-XPO complex. This sulfate, however, is only partially occupied despite 0.2M

Figure 3. Superposition, based on the positions of C α atoms, of ZD9331, ADP, and folate bound in the active site of FTHFS. FTHFS-ZD9331 complex is shown in the atom type and includes Phe384 and Trp412; folate is in brown and ADP in cyan. The adenine of ADP and the pterins of ZD9331 and folate are all sandwiched between the aromatic side chains of Phe384 and Trp412. The ZD9331 position is likely analogous to that of the substrate. For the folate ion, the para-aminobenzoic acid and the glutamate group are not positioned within the active site pocket but are pointing in the opposite direction, indicating a non-productive mode of binding. An interactive view is available in the electronic version of the article. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

concentration and likely binds after the translocation of the XPO takes place.

On the basis of this structure, a model of FTHFS-XPO-THF was built. The pterin moiety of

THF was placed parallel to its position in the ZD9331 complex and N¹⁰ in the vicinity of XPO. Rotations around single bonds positioned the carboxylates near positively charged residues K345

Figure 4. Stereoview of ZD9331 and XPO bound in FTHFS-ZD9331-XPO complex. $F_o - F_c$ electron density (contoured at 2σ level) is shown in green and corresponds to the partially occupied ZD9331 and XPO in the active site of subunit A. An interactive view is available in the electronic version of the article. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. Modeling of THF and XPO in the active site of FTHFS. This model is based on the structure of ZD9331·XPO complex and reflects that XPO translocates towards the center of the active site and rotates to have the formate moiety pointing towards the N¹⁰ atom. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

and R335, which side chain positions were not altered. These residues are highly conserved; they are present even in trifunctional human C1 synthase.

Discussion

The structures of FTHFS complexes with substrate analogs and intermediates allowed us to identify two sub-sites within the active site. One is located between the aromatic side chains of Trp412 and Phe384 and has high affinity for condensed ring systems (Fig. 3). The purine of ADP or ATP and the pterin of folates bind in this site. The second sub-site is the phosphate/sulfate binding site created by the side chains of Arg97 and Lys74 and the peptide nitrogen of Phe304. All four structures have this sub-site occupied by a tetrahedral anion; in crystals obtained by co-crystallization of FTHFS with ATP and formate the ligand is XPO. The presence of XPO, despite 1.6M sulfate concentration in the mother liquor, indicates the sub-site's high affinity for this ligand and that the bound ADP blocks its exchange with the solvent. The strong binding is generated by two additional H-bonds to the terminal oxygen of the formyl moiety formed by the nitrogen of Ala276 and the side chain of Arg97. However, the position of the formate moiety appears inaccessible to the N¹⁰ atom, which is located in the central part of the tetrahydrofolate molecule. Modeling shows that with the pterin moiety in the condensed ring binding site the XPO ion is too far from N¹⁰. Moreover, the formate moiety is pointing away from the folate and is buried with no potential access. Thus, it appears likely that upon ADP dissociation, the XPO ion translocates towards the center of the active site and rotates to have the formate moiety

pointing towards the folate (Figs. 4 and 5). The five H-bonds that stabilize the position of the ADP diphosphate generate the necessary binding affinity and XPO polarization. This shift of the XPO position may correlate with THF binding.

The FTHFS-folate complex was studied to elucidate the position of THF within the enzyme. PABA and the glutamate group of the folate are not positioned within the active site pocket, which indicates a non-productive mode of binding. This mode of binding may be due to the lack of the conformational change, as seen in the FTHFS-ADP·XPO complex, and suggests that THF binds in a similar mode in the absence of both ATP and formate.

The modeling shown in Figure 5 is based on what is seen in the FTHFS-ZD9331·XPO complex (Fig. 4). The N³ of ZD9331, which corresponds to the N¹⁰ of THF, is pointed towards the XPO, in position for nucleophilic attack. XPO is oriented so that the carbonyl is pointing towards the N³ of ZD9331, likely reflecting the situation in the catalytic complex. The mutants of Lys74⁵ and Arg97⁹ showed drastically reduced activity, which is consistent with the proposed central role of these residues in XPO generation and its retention in the active site. The observed sharing of the binding site by ATP and THF explains the reported substrate inhibition of FTHFS by high concentrations of THF, as the latter inhibits ATP binding.⁹ The overall catalytic mechanism by which FTHFS operates is proposed in Figure 6.

The studied here crystals of FTHFS-ADP·XPO complex were obtained by co-crystallization of the enzyme, ATP and formate. Their structure clearly shows that the first reaction, formation of XPO and

Figure 6. Proposed reaction mechanism for FTHFS. Formate, which is stabilized through hydrogen bonding from Arg97 and Ala276, attacks the γ -phosphate of ATP. Formylphosphate, the intermediate, is formed and ADP dissociates. Tetrahydrofolate, the third substrate, binds in the active site between Trp412 and Phe384, positioning its N¹⁰ towards the formylphosphate. Nucleophilic attack from N¹⁰ to the carbonyl of formylphosphate occurs, transferring the formyl group to tetrahydrofolate, forming the final products formyltetrahydrofolate and phosphate.

ADP, took place in the absence of THF. It is highly likely, however that this was a single turnover reaction. It is well established that XPO remains strongly bound to the enzyme¹⁸ and from the structure it is apparent that a next ATP molecule cannot bind in its presence. It must be so because otherwise FTHFS would effectively function as ATPase since XPO is not very stable. The second reaction, transfer of the formyl group to THF, is needed to remove XPO from the active site. This single turnover property of the first reaction led apparently to the misinterpretation of the kinetic data as a random ter ter mechanism.

When ATP, formate, and the antifolate were present, the enzyme again catalyzed the conversion ATP and formate to ADP and XPO. However, in the presence of the antifolate, ADP dissociated and ZD9331 bound utilizing a part of the active site previously occupied by ADP; thus the FTHFS·XPO·ZD9331 complex was formed. There is no evidence for the formation of quaternary complex FTHFS·ATP·formate·THF needed for the random ter ter mechanism, although all components were present. To the contrary, the formation of such a complex appears impossible because of a huge steric hindrance. Therefore it seems highly unlikely that all substrates can bind at the same time in the observed mode.

Our data do not suggest an explanation for the observation that the release of ADP is greatly accelerated by THF.¹⁸ One hypothesis that can be put forward is that THF binds first with its PABA moiety, initiating the release of ADP, whereas the pterin moiety inserts in between Phe348 and Trp412 only after ADP dissociation. However, folate, which has

the PABA moiety the same as THF, binds with the PABA outside the active site. Thus the presence of XPO and MgADP would have to be the factor that increases affinity for PABA binding.

The structural asymmetry of FTHFS·XPO·ZD9331 and FTHFS·folate complexes is small (Supporting Information Figs. S2A and S2B) despite the ligands presence in only one subunit. The empty active site is not blocked, nor interacts with any neighbors in the crystal lattice. These observations suggest that the lack of binding is due to negative cooperativity of the subunits. Some unresolved issues in FTHFS catalysis may be related to this phenomenon.

Materials and Methods

Chemicals

Salts, sugars, antibiotics, dithiothreitol (DTT), polyethylene glycols (PEGs), potassium maleic acid, tetrahydrofolic acid (THF), folic acid (folate), adenosine 5'-triphosphate (ATP), phenyl-sepharose CL-4B, were obtained from Sigma (St. Louis, MO). Heparin-Agarose was obtained from MP Biomedicals (Solon, OH), and ZD9331 was obtained from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK).

Expression and protein purification of FTHFS

FTHFS from *Moorella thermoacetica* was expressed into *Escherichia coli* strain Y1 bacterial systems as previously described²¹ and purified according to the Staben procedure with modifications.²² *E. coli* containing the FTHFS gene were cultivated in Luria-Bertani medium supplemented with 2.5 g/L dextrose, 2.5 g/L K₂HPO₄, and 15 μ g/mL tetracycline

Table I. Crystallographic Data and Refinement Statistics

Ligands	Native FTHFS	ADP/XPO	ZD9331/XPO	Folate
X-ray source	APS SER-CAT ID	APS SBC- BM	APS SER-CAT ID	APS SER-CAT BM
Wavelength (Å)	0.979	1.59	1.00	1.00
Number of frames	180	150	74	174
Oscillation range (°)	0.5	1.0	1.0	1.0
Crystal to detector distance (mm)	255	200	200	250
Temperature (K)	100	100	100	100
Space group	R32	P2 ₁ 2 ₁ 2	R32	R32
Unit cell dimensions				
<i>a</i> (Å)	161.20	91.17	162.37	160.99
<i>b</i> (Å)	161.20	212.97	162.37	160.99
<i>c</i> (Å)	256.92	53.44	258.07	256.61
Volume (Å ³)	5,781,836	1,037,715	6,000,656	5,759,745
% Solvent, Matthews Coefficient	53.72, 2.68	43.82, 2.21	56.02, 2.82	54.89, 2.75
Resolution range (Å) (outer shell)	40.9–2.2	50.0–2.50	50.0–2.67	50.0–3.0
	(2.28–2.20)	(2.59–2.50)	(2.78–2.67)	(3.11–3.00)
Average redundancy	4.8 (4.7)	3.8	4.5	9.7 (10.4)
Average <i>I</i> /σ (<i>I</i>)	10.3	8.0	9.4	7.8
Total number of reflections	312,543	141,119	360,101	248,751
Number of unique reflections	64,918	37,221	79,627	25,893
Completeness (%) (outer shell)	99.8 (99.9)	91.5 (64.8)	91.1 (87.4)	98.8 (100)
Total linear <i>R</i> -merge	7.6	8.5	4.9	16.5
<i>R</i> -value (%)	20.7 (refmac)	23.3 (CNS)	20.5 (CNS)	23.0 (CNS)
<i>R</i> _{Free} -value (%)	26.0 (refmac)	30.0 (CNS)	29.3 (CNS)	28.3 (CNS)
rmsd, bond lengths (Å)	0.02	0.02	0.02	0.01
rmsd, bond angles (deg.)	2.2	2.0	1.7	1.6
Ramachandran statistics				
Residues in most favored regions (%)	86.3	83.0	78.8	77.6
Residues in additional allowed regions (%)	13	15.4	20.1	21.4
Residues in generously allowed regions (%)	0.7	1.6	1.2	1.0
Residues in disallowed regions (%)	0.0	0.0	0.0	0.0
Average B factors for subunit A (ligands)	29.6	41.1 ^a	45.2	24.8
Average B factors for subunit B (ligands)	49.9	(51.0 ADP, 67.6 XPO)	(73.8 ZD)	(71.9 folate)
		36.8	48.2 XPO)	50.6
		(51.2 ADP, 59.5 XPO)	67.8	

^a Related to different crystal form.

and harvested by centrifugation. Cell pellets were washed with 50 mM potassium maleate buffer (KMB), pH 7.0, suspended in the same buffer, and disrupted by sonication using a Branson Sonifier 450 sonicator. All subsequent purification steps were performed at 4°C. Homogenate was centrifuged for 30 min at 10,000 rpm and clear yellow supernatant was separated from the cell debris. Streptomycin sulfate (0.4 mg/mL) was added to the extract and the mixture was incubated with gentle stirring at 4°C for 30 min. Nucleic acids and some contaminating proteins were removed by centrifugation at 10,000 rpm for 30 min. The crude extract was loaded onto a 12 cm × 1.5 cm, 15 mL bed volume, heparin-agarose column previously equilibrated with KMB. After washing with 30 mL KMB, proteins were eluted with a 0 to 0.4M linear gradient of KCl in KMB. Fractions were assayed for FTHFS activity²³ and those containing the highest activities were pooled. The active pool was then loaded onto a 12 cm × 1.5 cm, 15 mL bed volume, phenyl-sepharose column, pre-equilibrated with 0.7M ammonium sulfate in 50 mM KMB. The column was washed with equilibrium buffer and FTHFS was eluted with a

negative ammonium sulfate gradient of 0.7 to 0M in KMB. Fractions containing the greatest amount of activity were pooled and purity was confirmed by SDS-PAGE. Protein concentration was determined using the Lowry method.²⁴

Crystallization, crystal soaking, and co-crystallization

Crystals of FTHFS were grown as previously described²⁵ by hanging drop vapor diffusion in both high and low salt conditions. The high salt conditions contain 38 to 46% (w/v) saturated ammonium sulfate (AS), 1 mM DTT, and 1 to 3.5% (w/v) PEG 1000 or PEG 1450, in 50–75 mM KMB (pH 7.0–8.0). Low salt conditions contain 18 to 22% (w/v) PEG 6-8K, 0.2M AS, 1 mM DTT, 75 mM KMB pH 7.0–8.5.

Native crystals of FTHFS were grown in high salt conditions and were large hexagonal plates. They belonged to the *R*32 space group with unit cell dimensions *a* = 161.20 Å, *c* = 256.92 Å and two subunits per asymmetric part of the unit cell (APUC).

Crystallization of FTHFS in the presence of ATP (5 mM) and formate at high salt conditions described above yielded non-isomorphous crystals, which were

thick rectangular plates and belonged to the $P2_12_12$ space group with unit cell dimensions: $a = 91.17 \text{ \AA}$, $b = 212.98 \text{ \AA}$, $c = 53.44 \text{ \AA}$ and two subunits per APUC. Co-crystallization with folate (5 mM) at high salt conditions yielded crystals, isomorphous to the native crystals, which were thick hexagonal plates and belonged to the $R32$ space group with unit cell dimensions of $a = 160.97 \text{ \AA}$, $c = 256.61 \text{ \AA}$, with two subunits per APUC. Crystals grown at low salt conditions described above were soaked with ATP, formate, and ZD9331. Soaked crystals were isomorphous to the native crystals and were thick hexagonal plates which belonged to the $R32$ space group with unit cell dimensions of $a = 162.37 \text{ \AA}$, $c = 258.07 \text{ \AA}$, with two subunits per APUC.

X-Ray diffraction data collection and processing

All crystals were cryoconditioned by soaking in 20% ethylene glycol-enriched mother liquor and flash-frozen in a liquid N_2 vapor stream at 100 K. Each of the X-ray diffraction data sets were collected from a single crystal at the SER-CAT 22ID, SER-CAT 22BM, and SBC 19BM beamlines at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Argonne, IL. The data were indexed and processed with the HKL2000 software package²⁶; processing parameters and refinement statistics are summarized in Table I.

Structure determination and refinement

The native FTHFS structure reported was solved by molecular replacement with the Phaser²⁷ program from the CCP4 suite²⁸ using the lower resolution structure, PDB code 1EG7, as the initial search model. All other structures were solved by molecular replacement with CNS²⁹ using the reported here native structure, with PDB code 3PZX, as the initial search model. Models were rebuilt using TURBO software³⁰ while crystallographic refinements were carried out with the CNS²⁹ and Refmac5.³¹ Coordinates were superposed with LSQKAB program³² from the CCP4²⁸ suite. Geometry of the final models was verified using PROCHECK.³³ Figures 2A–B, 3–5, and Supporting Information Figures S4, S6–S7 were prepared using TURBO,³⁰ Supporting Information Figures S1, S2A–C, and S3A–B were prepared using Molscript³⁴ and Raster3D,³⁵ and Supporting Information Figures S5A–D were prepared using PyMOL.³⁶

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