Neutron diffraction reveals protonation states in pyridoxal-5′-phosphate-free and glycine external aldimine-bound serine hydroxymethyltransferase

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Short title: Neutron structures of SHMT reaction intermediates

Conflicts of interest

There are no conflicts of interest to declare.

Abbreviations

Tth – Thermus thermophilus; SHMT – serine hydroxymethyltransferase; THF – tetrahydrofolate; 5,10-MTHF – 5,10-methylene-tetrahydrofolate; 5MTHF – 5-methyl-tetrahydrofolate; hSHMT2 – human mitochondrial serine hydroxymethyltransferase; PLP- pyridoxal-5'-phosphate; FA – folinic acid.

Keywords: neutron crystallography, X-ray crystallography, serine hydroxymethyltransferase, protonation state, external aldimine.

ABSTRACT

Serine hydroxymethyltransferase (SHMT) is a critical enzyme in the one-carbon (1C) metabolism pathway catalyzing the reversible conversion of L-Ser into Gly and concurrent transfer of 1C unit to tetrahydrofolate (THF) to give 5,10-methylene-THF (5,10-MTHF), which is used in the downstream syntheses of biomolecules critical for cell proliferation. The cellular 1C metabolism is hijacked by many cancer types to support cancer cell proliferation, making SHMT a promising target for the design and development of novel small-molecule antimetabolite chemotherapies. To advance structure-assisted drug design, knowledge of SHMT catalysis is crucial, but can only be fully realized when the atomic details of each reaction step governed by the acid-base catalysis are elucidated by visualizing active site hydrogen atoms. Here, we used room-temperature neutron crystallography to directly determine protonation states in *Thermus thermophilus* SHMT (*Tth*SHMT), capturing protomer A in the *apo*-form lacking the coenzyme pyridoxal 5'-phosphate (PLP), and protomer B as a ternary complex with PLP–Gly-external-aldimine and (6*S*)-5-methyltetrahydrofolate (5MTHF). We observed protonation of the Schiff base nitrogen in PLP–Gly and neutrality of the catalytic Lys226 side chain in the ternary complex, whereas Lys226 is protonated and positively charged in the *apo*-active site. Furthermore, we obtained an X-ray structure of *Tth*SHMT in complex with the substrate THF, which binds identically as 5MTHF at the peripheral binding site. The unique structural and functional information provided by neutron crystallography, in combination with X-ray structures, can be employed in the rational design of SHMT inhibitors.

INTRODUCTION

Serine hydroxymethyltransferase (SHMT) is responsible for the reversible conversion of L-Ser into Gly and concurrent transfer of a one-carbon (1C) unit to tetrahydrofolate (THF) to produce 5,10-methylene-THF (5,10-MTHF). SHMT is a critical metabolic enzyme in the 1C metabolism pathway, which supplies building blocks for the downstream biosyntheses of DNA, RNA, amino acids, epigenetic regulation and redox homeostasis [1-7]. Additionally, in the absence of THF, SHMT can catalyze the cleavage of β-hydroxy amino acids, decarboxylation of aminomalonate, and racemization and transamination of L- and D-alanine [8,9]. The cellular 1C metabolism is highjacked by many cancer types, as its metabolic products are in high demand in the rapidly dividing malignant cells, supporting cancer cell proliferation and tumor growth [10-12]. Human mitochondrial SHMT2 (hSHMT2) plays an essential role in the 1C metabolic reprogramming – a recognized hallmark of cancer [13,14]. Overexpression of hSHMT2 has been linked to increased cancer aggressiveness and its ability to metastasize, leading in many instances to poor prognosis of the cancer treatment outcomes [15-17]. As a result, hSHMT2 is considered a promising target for the design and development of novel small-molecule antimetabolite chemotherapies [18-20].

Although inhibitors of varying chemical architectures targeting hSHMT2 have been recently developed [21-26] and some FDA-approved drugs, such as metformin [27], sertraline [28] and antifolate drugs [29], have been suggested for drug repurposing or as lead compounds to design hSHMT2-specific inhibitors, none have reached the clinic. To further the design of new hSHMT2 inhibitors, including those based on the enzyme's catalytic mechanism, it is of critical importance to understand SHMT catalysis by obtaining a detailed picture at the atomic level of each catalytic step. Because SHMT-catalyzed chemistry is governed by the general acid-base catalysis, the key is to define the protonation states of the enzyme active site, PLP coenzyme, and substrate THF, and to learn how they change along the catalytic reaction pathway. Therefore, positions of hydrogen (H) atoms, which define protonation states of specific chemical groups, must be determined with high accuracy and confidence. The only experimental technique capable of achieving this for functional, labile H atoms is macromolecular neutron crystallography [30,31]. This is because neutrons are scattered by atomic nuclei rather than by electron clouds that interact with X-rays. There is little electron density on labile H atoms connected to more electronegative

O or N atoms via highly polarized covalent bonds, leading to a very small X-ray scattering magnitude for H. Conversely, the neutron scattering of H and its isotope deuterium (D) is significantly stronger, being similar to that of C, N, and O, and does not depend on the electron density present on H or D. Hence, H/D positions can be accurately resolved with neutron crystallography [32-35]. Moreover, neutrons used for macromolecular crystallography experiments do not cause ionization of atoms like X-rays do, avoiding radiation damage to protein crystals.

We have been employing a bacterial SHMT from *Thermus thermophilus* (*Tth*SHMT) as a model enzyme to study hSHMT2 because the two proteins possess highly conserved active sites [36,37]. The appropriateness of this strategy has also been demonstrated in previous studies where other bacterial [38,39] and plant SHMTs [40,41] were used to mimic hSHMT2. Notably, *Tth*SHMT affords high-quality crystals amenable to soaking with various substrates and inhibitors. This allows capturing the enzyme at different catalytic reaction stages and enables neutron and X-ray crystallographic studies of stable intermediate states at near-physiological (room) temperature. As in all PLP-dependent enzymes, the *Tth*SHMT holoenzyme active site contains the PLP coenzyme covalently bonded to the catalytic Lys226 (Lys280 in hSHMT2) through a Schiff base linkage called the internal aldimine. In our studies [36,37], we observed protonated (positively charged) pyridine N1, deprotonated (negatively charged) phenolic O3′, and non-protonated (neutral) Schiff base N_{SB} atoms in the PLP internal aldimine. PLP is caged by five His residues – His122, His125, His200, His225, and His312 (His171, His174, His254, His279, and His380 in hSHMT2, respectively) – that are all neutral and identical tautomers, with non-protonated Nδ1 and protonated Nε2. These His protonation states remain invariant upon ligand binding. Interestingly, the substrate amino acid, L-Ser, has two binding sites. It initially binds at the peripheral binding site near the enzyme surface, where THF, its analogs and inhibitors normally are located and then it moves into the active site interior to bind at the cationic binding site near PLP. L-Ser was found as a zwitterion when bound to the peripheral binding site indicating its amino group must be deprotonated to activate it for the subsequent nucleophilic attack on the Schiff base C4′, which results in the production of the PLP-L-Ser external aldimine through the transaldimination reaction. Moreover, we observed that the active site Glu53 (Glu98 in hSHMT2) can change its protonation state from being deprotonated (negatively charged) in the holoenzyme to becoming protonated (neutral) when a THF analog folinic acid binds. We thus proposed that Glu53,

strategically located in close proximity to PLP-L-Ser and THF, can act as a universal acid-base catalyst in the direct displacement mechanism for the THF-dependent reaction catalyzed by SHMT [37], in contrast to earlier catalytic mechanisms that suggested the involvement of as many as six different general acids and bases [42-45].

Building upon our previous studies [36,37], we present here a 2.1 Å resolution room-temperature neutron structure of *Tth*SHMT in complex with PLP-Gly external aldimine and (6*S*)-5-methyltetrahydrofolate (5MTHF), a substrate analog of THF. The current *Tth*SHMT/PLP-Gly/5MTHF neutron structure resembles a reaction intermediate after the L-Ser side chain CH_2-OH moiety is transferred from the PLP-L-Ser external aldimine to THF. In addition, because the chemical reactivities of the two protomers in the *Tth*SHMT homodimer are not identical in crystals, as discussed previously [36], the other protomer was captured in the *apo*-form, lacking both PLP and 5MTHF, in the same neutron structure (Figure 1A). Moreover, we obtained a room-temperature X-ray structure of *Tth*SHMT with PLP in the internal aldimine state complexed with THF substrate to uncover structural differences between the enzyme active sites bound to THF or its analogs. In the neutron structure of *Tth*SHMT/PLP-Gly/5MTHF complex, we consistently observed invariance of the active site His protonation states and tautomeric forms. The catalytic Glu53 was found to be deprotonated as in the holoenzyme. In the PLP-Gly external aldimine, the Schiff base N_{SB} was observed as protonated (positively charged), whereas the freed Lys226 side chain adopted the non-protonated (neutral amine) state. Protonation states of the most active site residues were identical in the *apo*-protomer to those observed in the PLP-Gly/5MTHF-bound protomer, except for the catalytic Lys226 which was observed in the protonated (positively charged ammonium) state. The current neutron structure of *Tth*SHMT depicting protonation states in the *apo*- and PLP-Gly/5MTHF-bound active sites and our previous neutron structures allow us to follow the H atom movements and structural reorganizations along the SHMT functional pathway.

RESULTS

Structural changes upon Gly and 5MTHF binding to *Tth*SHMT

Each *Tth*SHMT protomer can be divided into a large domain spanning residues 33-284 and a small domain consisting of residues 285-407 (Figure 1A). Analysis of the protomer B active site

conformation relative to that observed in the *apo*-state protomer A reveals that a portion of the small domain with residues 313-360 undergoes a significant structural rearrangement leading to the full closure of the active site. This conformational change is accomplished through a shift in the position of the gating loop (residues 342-356) by as much as 4 Å, accompanied by smaller movements of the adjacent secondary structure elements (Figure 1B). The ability of the active site in *Tth*SHMT to cycle between the open and closed conformations is vital for the enzyme function. For example, we previously showed [37] that protomer A in the holoenzyme is unreactive towards L-Ser substrate and does not bind FA because the gating loop conformational dynamics is compromised by the crystal packing interactions.

A crystal of the holoenzyme with the PLP in the internal aldimine form in both protomers was soaked with a mixture containing both Gly amino acid and 5MTHF to produce the *Tth*SHMT/PLP-Gly/5MTHF complex. The 2.1 Å neutron diffraction data were collected from this crystal at room temperature, and the neutron structure was jointly refined with a 1.7 Å room-temperature X-ray data obtained from another crystal treated in the same fashion (Table 1). *Tth*SHMT crystallizes in a monoclinic unit cell (space group $P2_1$), with the enzyme homodimer present within the asymmetric unit (Figure 1A). Protomer B afforded the PLP-Gly external aldimine and 5MTHF bound in the active site, where Gly is covalently attached to PLP through a Schiff base linkage and its C-terminal carboxylate is placed within the cationic binding site. The peripheral binding site harbors the 5MTHF molecule (Figure 1B). Thus, protomer B in the current *Tth*SHMT/PLP-Gly/5MTHF represents a ternary enzyme complex, having the PLP-Gly intermediate and a THF analogue occupying the active site. Protomer A, however, was found in the *apo*-form lacking PLP co-enzyme completely. Two sulfate anions were observed in the active site of protomer A, occupying the cationic and phosphate binding sites. This observation was not unexpected because the protomers in these TthSHMT crystals have different chemical reactivities due to packing effects as shown previously [36,37], and we observed the identical result when Gly and folinic acid (FA), a THF analogue, were soaked into a *Tth*SHMT holoenzyme crystal [37].

Protonation states in PLP-Gly/5MTHF-bound active site

Binding of the amino acid, Gly, results in its nucleophilic attack on the C4′ Schiff base carbon atom covalently linked to the catalytic Lys226 ε-amino group, ultimately leading to the

release of the lysine side chain and production of the PLP-Gly external aldimine, in which the Schiff base covalent linkage (C4′=N_{SB}) is now formed between the PLP C4′ and Gly amino group (Figure 1C). The reaction can proceed further to give the PLP-Gly quinonoid intermediate according to the previously proposed catalytic mechanism [37]. We, however, believe that *Tth*SHMT/PLP-Gly/5MTHF structure contains the external aldimine, as evidenced below. The production of PLP-Gly and the subsequent binding of 5MTHF do not perturb the protonation states of the active site residues that remain unchanged compared to the holoenzyme [36]. Importantly, all active site histidine residues remain neutral, with Nε2 nitrogen atoms protonated and Nδ1 not protonated. The N1 pyridine nitrogen of PLP-Gly maintains its protonated positively charged state also found in the internal aldimine (Figure 2A). N1 is H bonded with the carboxylate side chain of Asp197 with D···O and N···O distances of 1.6 and 2.6 Å, respectively. Asp197 is anchored by three additional H bonds with the side chains of Asn98 and His125, and with the Ala199 main chain. The O3′ oxygen atom is deprotonated, maintaining its phenolate status, as in the structures with PLP internal aldimine. O3′ makes a conventional H bond with the Ser172 side chain (D···O and O···O distances of 1.8 and 2.7 Å, respectively) and a weaker unconventional C-H···O bond with Cδ2 of His200 imidazole (H···O and C···O distances of 2.4 and 3.3 Å, respectively). His200 is further H bonded to His312 that faces its own main chain amide ND, terminating this H bonding network (Figure 2B). The specific orientation of the imidazole ring of His200 allows its protonated Nε2 atom to H bond with the PLP-Gly carboxylate group, whose position is stabilized through a salt bridge with the positively charged Arg358 and an extra H bond with Tyr61* side chain (Figure 2C, * denotes the residue belongs to protomer A).

The Schiff base nitrogen N_{SB} of PLP-Gly was found to be protonated, hence, positively charged. Thus, N_{SB} gains an H atom in the external aldimine when PLP reacts with Gly, because the Schiff base was observed neutral (non-protonated) in the PLP internal aldimine state, unaffected by L-Ser or FA binding at the peripheral binding site [36,37]. Interestingly, the N_{SB}-H group did not undergo H/D exchange, supported by the H occupancy refining to 100% and a peak in the negative omit nuclear density map (Figure 2D). H atoms appear as troughs in the negative nuclear density maps because the coherent neutron scattering length of H is –3.74 fm, whereas that of D is +6.67 fm. In addition, H/D exchange did not occur for the adjacent Ser172 side chain hydroxyl, supporting a notion that this side of PLP-Gly is not accessible by solvent. Conversely,

the C4′-H bond, located on the opposite side of PLP-Gly, fully exchanged with D, supported by the D occupancy refining to 100% and a peak in the omit nuclear density map (Figure 2A and 2D). This observation indicates C4′-H bond may be more acidic in the protonated external aldimine than in the neutral internal aldimine because H/D exchange of C4′-H has not been detected in previous *Tth*SHMT neutron structures [36,37]. The N_{SB}-H bond faces O3′ making a 1.6 Å H bond that completes a six-membered cycle stabilizing the position of the Schiff base (Figure 2A). The N_{SB}-H···O3′ H bond formation is a result of the protonated Schiff base C4′=N_{SB} double bond being nearly co-planar with the pyridine heterocycle in the PLP-Gly external aldimine. C4′=N_{SB} rotates below the pyridine plane on the *re* face with the dihedral angle of -8°, having rotated by at least 40° from the out-of-plane geometry observed for the neutral Schiff base of the PLP internal aldimine where the corresponding dihedral angles were measured at 29-38° [36,37] (Figure 2E). Such tilt of the Schiff base was even greater in hSHMT2 (67-73°) [36] and AAT (46°) [46] internal aldimine structures. Similar to *Tth*SHMT/PLP-Gly/5MTHF, this dihedral angle was much reduced in the PLP-Gly external aldimine of hSHMT2 measuring 2° and 9° on the PLP *si* face for two protomers, and -13° and -14° on the PLP *re* face for the other two protomers in the enzyme homotetramer [37]. The Schiff base rotated further to -28° on the PLP *re* face in AAT external aldimine complex with α-methylaspartate where N_{SB} was also found to be protonated. Evidently, protonation of N_{SB} promotes the Schiff base conformational change to be nearly co-planar with pyridine or move further to the PLP *re* face.

The catalytic Lys226 released from the covalent linkage with PLP upon the PLP-Gly formation is positioned on the *si* face, above the Schiff base, and only 3.2 Å away from C4′ (Figure 2F). The Lys226 ε-amino group is a neutral ND_2 and is oriented in such a way that its D atoms are directed towards the PLP plane. In this orientation Lys226 ND_2 makes no H bonds with the nearby amino acid residues. The ε-amino nitrogen is 3.5 Å away from the hydroxyl group of Thr223 and Tyr51*, making a weak H bond with the phenolic oxygen of Tyr51* with the N-D···O distance of 2.6 Å. However, Lys226 ND_2 is engaged in the stabilizing N-D···π interactions with the PLP external aldimine having distances of 3.2-3.4 Å. Importantly, the neutrality of Lys226 ε-amino group points to our conclusion that PLP-Gly is indeed the external aldimine rather than quinonoid intermediate, that is produced when Lys226 abstracts an H from Gly Cα in the subsequent reaction step to become protonated ND_3^+ (see Figure 5C in ref. 37). The catalytic Glu53* side chain is

observed as a deprotonated, negatively charged, carboxylate (Figure 2G) that is H-bonded to N10 of 5MTHF (D···O and N···O distances of 2.2 and 3.0 Å, respectively) and is connected to His122 through a water-mediated interaction. The protonation state of Glu53* remains unchanged compared to the holoenzyme structure and the L-Ser *pre*-Michaelis state complex [36].

5MTHF is bound at the peripheral binding site in a similar fashion as does FA in the *Tth*SHMT/FA complex reported previously [37], selecting for the C6-(*S*) diastereomer of 5MTHF (Figure 1C). Unlike the THF substrate, 5MTHF is substituted at the N5 position with a methyl group, which points into a pocket surrounded by His122, Glu53*, Tyr61*, and the carboxylate group of PLP-Gly (Figure 2H). Similar to FA, 5MTHF binds as the N3-protonated tautomer creating a bridge with the main chain atoms from Gly121 and Leu123 (Figure 2G). Additionally, the N2′ amine is H-bonded with the main chain carbonyl of Leu117 and a water molecule that connects it to Asn342. The side chain of Asn342 is locked in position by an H-bond with N8 and a weak contact with N1, which is not protonated in the observed 5MTHF tautomer.

Protonation states in *apo*-active site

The active site of protomer A in *Tth*SHMT/PLP-Gly/5MTHF complex is observed in the *apo*-form, with no PLP present, making it the first neutron structure of any PLP-dependent enzyme in the PLP-free state, although several such X-ray structures of SHMTs have been previously reported [37,47,48]. In the *apo*-*Tth*SHMT active site, two sulfate anions (SO_4^{2-}) template the positions normally occupied by the PLP in the phosphate binding site and by the amino acid substrate in the cationic binding site (Figure 3A and 3B). The former SO_4^{2-} makes water-mediated interactions with Lys226* and direct H bonds with Ser93*, Gly94*, and Ser95*. The latter SO_4^{2-} makes a salt-bridge with Arg358* and additional H bonds with Ser31*, His200*, Lys226*, and Tyr61. The catalytic Lys226* is protonated, with a positively charged, ammonium ND_3^+. Its ε-ammonium group is hydrated by three water molecules that bridge it to Asp197*, that anchors the pyridine moiety of PLP in both the internal and external aldimine forms, and the SO_4^{2-} bound in the PLP phosphate binding site. Importantly, all active site His residues, including His122*, His125*, His200*, His225*, and His312*, are neutral as seen in all previous neutron structures of *Tth*SHMT [36,37]. The catalytic Glu53 is deprotonated, negatively charged, compensating the positive charge on the nearby Lys226*.

Binding of THF

To visualize the binding of the actual substrate in the peripheral binding site and compare its pose with those of FA and 5MTHF molecules, we determined a room-temperature X-ray structure of *Tth*SHMT in complex with THF at 1.8 Å resolution (Table 2). As in the *Tth*SHMT/FA complex, THF is found only in protomer B, and the PLP internal aldimine is retained in both protomers. Conversely, the cationic binding site in *Tth*SHMT/THF is occupied by SO_4^{2-} whereas the acetate molecule was observed at this site in the *Tth*SHMT/FA complex. The electron density reveals that only the C6-(*S*) diastereomer of THF is bound at the peripheral binding site in the *Tth*SHMT/THF complex (Figure 4A). The N5 of THF, that does not have another chemical group covalently attached to it, such as a methyl in 5MTHF or an aldehyde in FA, forms a direct H bond of 2.7 Å with the SO_4^{2-} anion positioned at the cationic binding site. The closely located Glu53* is H bonded with N10 of THF, with the distance of 3.3 Å, and also weakly interacts with N5 that is just 3.7 Å away (Figure 4B). THF has an identical binding pose as was observed for 5MTHF and FA (Figure 4C). The SO_4^{2-} position is stabilized by a salt bridge with Arg358 and a network of additional H bonds and water-mediated interactions that are similar to those observed in the holoenzyme structure [36]. The Schiff base in *Tth*SHMT/THF is rotated by 18° above the PLP pyridine plane on the *si* face. Although in this conformation the Schiff base is slightly less out-of-plane than in previous structures of the holoenzyme and the *Tth*SHMT/FA complex, the dihedral angle is in good agreement with our previous DFT calculations that showed the energy minimum for the Schiff base rotation around the PLP pyridine ring is 21°. We therefore suggest that N_{SB} is neutral in *Tth*SHMT/THF, as observed for the PLP internal aldimine in the previous neutron structures [36,37].

DISCUSSION

Numerous biochemical transformations are facilitated through proton transfer events as governed by general acid-base catalysis, shaping the electrostatics of the enzyme active site to promote a particular chemical reaction. In addition, ligands are capable of inducing changes in protonation states upon binding, reconfiguring the electrostatics of the active sites. Therefore, knowledge of the H atom positions and their movements is of paramount importance for the

mechanistic enzymatic studies and for structure-assisted drug design. Within the framework of our study aimed at elucidating the atomic details of SHMT catalysis, we have now obtained five unique neutron structures along the reaction pathway, that were made possible due to different binding properties and chemical reactivities of the *Tth*SHMT protomers within the crystal lattice. These include: 1) the *apo*-active site containing no bound PLP (this work); 2) PLP in the internal aldimine form [36]; 3) the pre-Michaelis complex with L-Ser amino acid substrate bound at the peripheral binding site [36]; 4) a ternary complex with bound FA at the peripheral binding site, acetate molecule bound at the cationic binding site, and PLP in the internal aldimine form [37]; and, 5) a reaction intermediate containing PLP-Gly external aldimine and 5MTHF (this work).

In the *apo*-active site of *Tth*SHMT, the catalytic Lys226 has a well-defined extended conformation with its side-chain terminal ε-amino group being protonated (ND_3^+) and hydrated by three water molecules. To generate the holoenzyme, Lys226 must react with free PLP (an aldehyde) producing the internal aldimine form, a reaction that requires the Lys226 side chain to be deprotonated. Based on our current neutron structure, Lys226 deprotonation can be accomplished by Asp197 through the Grötthus mechanism. Asp197 could then donate this proton to the pyridine nitrogen of PLP, ultimately resulting in the internal aldimine as observed in our holoenzyme neutron structure [36]. In both the *apo*-active site and holoenzyme, all active site His residues are observed as singly protonated, neutral, and identical tautomers having Nε2, but not Nδ1, protonated. Moreover, the catalytic Glu53 is found as a deprotonated carboxylate in both structures. Of note, *apo*-hSHMT2 functions to block the ubiquitylase activity of protease BRCC36 by binding to the deubiquitylating BRCC36 isopeptidase complex (BRISC) to promote inflammatory cytokine signaling [49,50]. The amino acid substrate, L-Ser, binds to the SHMT active site in two stages – first, it enters through the peripheral binding site where it was found in the zwitterionic form with the N-terminal amine protonated and the C-terminal carboxyl deprotonated, and second, it proceeds further inside and is captured by Arg358, making a salt-bridge with the substrate's carboxylate [36]. For the amino acid substrate to react with the Schiff base, its amino group has to be deprotonated, which may be achieved by the N_{SB} nitrogen. This transaldimination reaction leads to the conversion of the internal aldimine into the external aldimine form of PLP, having a protonated N_{SB} and an almost coplanar conformation of the Schiff base with the PLP pyridine ring, as observed in our current neutron structure. During this conversion, Lys226 is released from its covalent linkage with PLP and remains situated just above

the pyridine ring plane. The Lys226 side chain is not protonated and neutral (ND_2). The Lys226 ε-amino group can then deprotonate Cα of the external aldimine to become a positively charged ND_3^+ generating the quinonoid intermediate, which we did not detect occurring in our neutron structure. Notably, in our neutron structures, we observed that Glu53 is capable of undergoing protonation/deprotonation cycles and is correctly positioned to move protons between the amino acid and THF substrates and intermediates along the reaction pathway. Therefore, the Glu53 residue was proposed to play a central role in both THF-independent and THF-dependent chemical transformations, acting as the general acid-base catalyst to drive SHMT catalysis [36]. Because the active sites are conserved in various SHMT enzymes, we previously suggested that the observations made in *Tth*SHMT are transferrable to hSHMT2, implying the catalytic mechanism is identical for the human enzyme.

CONCLUSION

By using neutron protein crystallography at near-physiological (room) temperature and capitalizing on the differences in the ligand binding properties and reactivity of the two protomers in *Tth*SHMT crystals, we succeeded in visualizing the protonation states in the *apo*-active site and the PLP-Gly external aldimine with bound 5MTHF, a THF substrate analog. The PLP-Gly external aldimine is protonated at both pyridine N1 and Schiff base N_{SB} atoms, while having the deprotonated phenolate O3′. Lys226 side chain ε-amino group is neutral, providing indirect evidence that PLP-Gly is indeed the external aldimine rather than a quinonoid intermediate. Similarly, all the active site His residues are neutral, whereas Glu53 is negatively charged. Conversely, Lys226 is protonated, positively charged, in the *apo*-active site. The unique structural and functional information provided by the current and previous neutron structures of *Tth*SHMT can be mapped onto hSHMT2 and employed in the rational design of SHMT inhibitors, targeting *apo*-enzyme and different stages of the catalytic reaction, which will be further aided by determining the neutron structures of SHMT complexes with known inhibitors [22,25,26,51-53].

MATERIALS AND METHODS

General information

Columns for protein purification were purchased from Cytiva (Piscataway, New Jersey, USA). His-tagged Tobacco Etch Virus (TEV) protease was produced in-house. Crystallization reagents and supplies were purchased from Hampton Research (Aliso Viejo, California, USA). Crystallographic supplies for crystal mounting and X-ray and neutron diffraction data collection at room temperature were purchased from MiTeGen (Ithaca, New York, USA) and Vitrocom (Mountain Lakes, New Jersey, USA). Glycine (cat. no. G7126), THF (a mixture of diastereomers, cat. no. T3125), and 5MTHF (a mixture of diastereomers, cat. no. M0132) were purchased from Millipore Sigma (St. Louis, Montana, USA).

Expression and purification. A detailed procedure for the expression and purification of *Tth*SHMT was published elsewhere [36,37]. The kanamycin-resistant pJ411 plasmid (ATUM, Newark, CA) encoding *TthSHMT* and an N-terminal linker containing a His_6-tag, a 34 amino acid long linker sequence and a TEV protease cleavage tag was transformed into the *E.coli* BL21(DE3) expression vector and the cells were grown in Luria-Bertani (LB) media with 50 μg/mL kanamycin at 37°C to an optical density of 0.8–1.0. The cell cultures were typically induced with 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) and the protein expressed overnight at 22 °C. The next day the cells were centrifuged, the cell pellets were resuspended in the lysis buffer made with 50 mM sodium phosphate pH 7.5, 500 mM NaCl, and 10 mM imidazole at a ratio of 5 mL of the buffer per gram of wet cell paste. Lysozyme was added to the resuspended cells at a concentration of 0.1 mg/mL and stirred on ice for 30 min. The cells were disrupted by sonication and the lysate was clarified by centrifugation at 30,000 *g*. The His-tagged *Tth*SHMT was purified via affinity chromatography using a HisTrapFF (5mL) nickel column equilibrated with 20 mM HEPES pH 7.5, 100 mM NaCl, and 10 mM imidazole. The protein eluted as a single peak from the column using a linear gradient of 20 mM HEPES pH 7.5, 100 mM NaCl, and 500 mM imidazole. TEV protease (1 mg TEV protease/100 mg of tagged protein) was added to purified *Tth*SHMT and the mixture was dialyzed against 20 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM EDTA overnight at room temperature. The resultant solution containing TEV protease, untagged *Tth*SHMT and the remaining unreacted tagged protein was loaded onto a HisTrapFF (5 mL) nickel column and untagged *Tth*SHMT was collected in the flow-through. Pure *Tth*SHMT was dialyzed against 40 mM NaOAc pH 5.4 and 1 mM PLP, concentrated to ~20 mg/mL and stored in 20% (v/v) glycerol at -30 °C.

Crystallization, soaking, and H/D-exchange. For crystallization experiments, frozen *Tth*SHMT was thawed and dialyzed overnight against 40 mM NaOAc pH 5.4 and 1 mM PLP to remove the glycerol cryoprotectant. *Tth*SHMT was then crystallized by sitting drop vapor diffusion methodology using 40 mM NaOAc pH 5.5, 1 M $(NH_4)_2SO_4$, and 0.5 M Li_2SO_4 as the precipitant solution at 16 °C. To obtain large crystals the initial aggregates were crushed, and new crystallization drops were microseeded with a seeding tool from Hampton Research in 9-well glass plates and sandwich box setups. Because THF analogs do not bind to *Tth*SHMT in high concentrations of salt, a crystal was transferred to a fresh drop containing 40 mM NaOAc pH 5.5 and 15% PEG 4000 and soaked overnight to remove most of sulfate. The crystal was then moved into another drop containing a soaking solution with 40 mM NaOAc pH 5.5, 15% PEG 4000, 0.5 M glycine and 10 mM 5MTHF on the next day. The crystal was kept in the latter soaking solution overnight before being mounted in a 2 mm-inner diameter quartz capillary containing a liquid plug made of 40 mM NaOAc pH 5.5, and 15% PEG 4000 in 99.8% D_2O to perform H/D-vapor exchange. Crystal soaks to obtain the *Tth*SHMT/THF complex were carried out in a similar manner, except the final soaking solution contained 40 mM NaOAc pH 5.5, 15% PEG 4000, and 10 mM THF.

X-ray diffraction data collection and structure refinement. Room temperature X-ray diffraction data collection on *Tth*SHMT/PLP-Gly/5MTHF and *Tth*SHMT/THF crystals were performed on a Rigaku HighFlux HomeLab instrument equipped with a MicroMax-007 HF X-ray generator, Osmic VariMax optics, and a DECTRIS Eiger R 4 M detector at ORNL according to previously published procedures [36,37]. The data were indexed and integrated using the CrysAlis Pro software package (Rigaku, The Woodlands, TX), and the data were reduced and scaled in the AIMLESS program in the CCP4 software suite [54,55]. The X-ray structures were solved by molecular replacement in PHASER [56] using phases from PDB code 8SUJ and refined with phenix.refine in the PHENIX suite [57,58]. The room temperature X-ray structure of *Tth*SHMT/PLP-Gly/5MTHF was subsequently used in joint X-ray/neutron refinement. Ligand restraints for PLP-Gly external aldimine, 5MTHF and THF were generated with eLBOW [59] using geometry optimized by quantum chemical calculations in Gaussian16 [60] at B3LYP/6-31 g(d,p) level of theory. The X-ray diffraction data collection statistics are presented in Tables 1 and 2.

Neutron diffraction data collection. Neutron diffraction was tested at room temperature on the IMAGINE [61-64] instrument located at the High Flux Isotope Reactor (Oak Ridge National Laboratory) using the broad bandpass functionality with neutron wavelengths between 2.8 and 10 Å. Neutron quasi-Laue diffraction data from a 1 mm^3 crystal of the *Tth*SHMT/PLP-Gly/5MTHF complex were collected at room temperature using the LADI diffractometer [65] at the Institut Laue-Langevin (ILL) in Grenoble according to published procedures [66]. A neutron wavelength range ($\Delta\lambda/\lambda$=30%) of 2.88-3.74 Å was used for data collection with diffraction data extending to 2.1 Å resolution. The crystal was held stationary at different φ (vertical rotation axis) for each exposure. A total of 39 images were recorded with an average exposure time of 12 hours per image from 3 different crystal orientations. The neutron data were processed using the Daresbury Laboratory LAUE suite program *LAUEGEN* modified to account for the cylindrical geometry of the detector [67,68]. The program *LSCALE* [69] was used to determine the wavelength-normalization curve using the intensities of symmetry-equivalent reflections measured at different wavelengths. No explicit absorption corrections were applied. The data were merged and scaled using *SCALA* [70]. Neutron data collection can be found in Table 1.

Joint X-ray/neutron (XN) refinement. Joint XN refinement of the *Tth*SHMT/PLP-Gly/5MTHF complex was carried out using *nCNS* [71,72], a patch of the Crystallography & NMR Systems (CNS) [73] software suite in the same manner as for our previous *Tth*SHMT structures [36,37]. A single rigid body refinement was the first step in the refinement procedure. Subsequently, several rounds of atomic position, atomic displacement parameter, and D atom occupancy refinements were performed. In between the rounds of the joint XN refinement, the structure was visualized in the molecular graphics program COOT [74] to confirm correct side chain modeling and direct the rotation of side chain hydroxyl, thiol, and ammonium groups as well as water molecules to construct accurate H bonding networks. Water molecules were modeled and refined as D_2O due to H/D-vapor exchange. *Tth*SHMT/PLP-Gly/5MTHF was modeled with H atoms at non-exchangeable positions because hydrogenous protein was used in the experiment, while labile positions were initially modeled as D atoms. After D atom occupancy refinement, the exchangeable sites were modeled based on individual site occupancies, where an occupancy of –0.56 is indicative of a pure H atom and an occupancy of 1.00 reflects a pure D atom. Sites partially occupied by both H and D atoms were given two atom records with the partial occupancies adding up to 1.00. The percentage of D atom occupancy at a specific site is calculated according to the

following formula: % D = (occupancy(D) + 0.56)/1.56. Joint X-ray/neutron refinement statistics can be found in Table 1.

Author contributions

V.N.D., R.S.P. and A.K. designed the study. V.N.D. expressed, purified and crystallized the proteins. V.N.D. and A.K. collected and reduced X-ray diffraction data and refined the structures. M.P.B. collected and reduced neutron diffraction data. V.N.D. and A.K. refined the joint XN structure. V.N.D., R.S.P., and A.K. wrote the paper with contributions from all authors.

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Data Availability

The data that support the findings of this study are openly available in the Protein Data Bank at https://www.rcsb.org/, reference numbers PDB: 9O5G, PDB: 9O50.

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Table 1. Crystallographic data collection and refinement statistics for the room-temperature joint X-ray/neutron structure of *Tth*SHMT/PLP-Gly/5MTHF complex. Values in parentheses are for the highest-resolution shell.

	***Tth*SHMT/PLP-Gly/5MTHF** Room Temp. PDB ID 9O5G	
Data collection:	**Neutron**	**X-ray**
Beamline/Facility	LADI-DALI (ILL, Grenoble)	Rigaku HighFlux HomeLab
Space group	$P2_1$	
Cell dimensions:		
a, *b*, *c* (Å); α, β, γ (°)	58.9, 83.7, 95.5; 90.0, 91.6, 90.0	
Resolution (Å)	50.7 – 2.10 (2.21-2.10)	95.5 – 1.70 (1.76 – 1.70)
No. reflections measured	214548 (26755)	420311 (29559)
No. reflections unique	40997 (4890)	101159 (9812)
R_{merge}	0.224 (0.495)	0.089 (0.487)
R_{pim}	0.104 (0.192)	0.046 (0.326)
$CC_{1/2}$	0.989 (0.718)	0.989 (0.667)
$\langle I / \sigma I \rangle$	8.3 (2.5)	21.7 (1.8)
Completeness (%)	76.6 (62.7)	99.3 (96.3)
Redundancy	5.2 (5.5)	4.2 (3.0)
Wilson B-factor (Å)	31.5	15.2
Refinement:	**Joint XN**	
Resolution (neutron, Å)	40.0 – 2.10	
Resolution (X-ray, Å)	40.0 – 1.70	
Data rejection criteria	no observation & |F|=0	
Sigma cut-off	2.50	
No. reflections (neutron)	36440	
No. reflections (X-ray)	91923	
R_{work} / R_{free} (neutron)	0.292/0.327	
R_{work} / R_{free} (X-ray)	0.181/0.195	
R_{work} / R_{free} (joint XN)	0.225/0.249	
No. atoms		
Protein, including H and D	12513	
5MTHF	56	
PLP-Gly	31	
Sulfate	25	
Water	1191 (i.e. 397 D_2O molecules)	
B-factors		
Protein	17.7	
5MTHF	47.8	
PLP-Gly	17.2	
Sulfate	54.8	
Water	33.0	
R.M.S. deviations		
Bond lengths (Å)	0.007	
Bond angles (°)	1.04	
All-atom clash score	1.03	
Ramachandran Stats. (%)		
Favored	97.50	
Allowed	2.38	
Outliers	0.12	

Table 2. Crystallographic data collection and refinement statistics for the room-temperature X-ray structure of *Tth*SHMT/THF complex. Values in parentheses are for the highest-resolution shell.

	***Tth*SHMT-THF** Room Temp. PDB ID 9O50
Data collection:	**X-ray (in-house)**
Diffractometer	Rigaku HighFlux, Eiger R 4M
Space group	$P2_1$
Wavelength (Å)	1.5406
Cell dimensions:	
a, *b*, *c* (Å)	58.9, 83.4, 95.3
α, β, γ (°)	90.0, 91.6, 90.0
Resolution (Å)	95.26 – 1.80 (1.87 – 1.80)
No. reflections unique	84515 (8255)
R_{merge}	0.092 (0.369)
R_{pim}	0.059 (0.309)
$CC_{1/2}$	0.967 (0.741)
$\langle I / \sigma I \rangle$	16.1 (2.1)
Completeness (%)	98.9 (96.6)
Redundancy	3.1 (1.9)
Wilson B-factor (Å)	16.2
Refinement:	
R_{work} / R_{free}	0.144/0.175
B-factors	
Protein	19.1
THF	22.5
Sulfate	51.8
Water	31.5
R.M.S. deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.228
All atom clash score	2.52
Ramachandran Stats. (%)	
Favored	97.48
Allowed	2.52
Outliers	0.0

Figure Legends

Figure 1. Three-dimensional structure of *Thermus thermophilus* serine hydroxymethyltransferase (*Tth*SHMT), pyridoxal-5′-phosphate (PLP) coenzyme and 5-methyl tetrahydrofolate (5MTHF). (**A**) Cartoon representation of *Tth*SHMT homodimer showing apo-protomer A and PLP-Gly/5MTHF-bound protomer B (PDB ID 9O5G). Large domains are colored red and blue; small domains are pink and light blue. (**B**) Superposition of protomer A and B revealing conformational changes of the gating loop and nearby regions. (**C**) Chemical structures of the coenzyme and 5MTHF. Figure is generated using PyMol (Schrödinger, Inc.) and ChemDraw (Revvity Signals Software, Inc.).

Figure 2. Neutron scattering length density maps and interactions for the protomer B active site in *Tth*SHMT/PLP-Gly/5MTHF complex. The $2F_O$-F_C map shown as light brown mesh is contoured at 1 σ level, omit F_O-F_C map in red mesh is contoured at 3 σ level. PDB ID 9O5G. (**A**) Binding of PLP-Gly external aldimine. (**B**) H-binding network near His200. (**C**) Interactions of PLP-Gly carboxylate group. (**D**) Protonation of the PLP-Gly external aldimine. Negative omit F_O-F_C map showing lack of H/D exchange of N_{SB} (i.e., N_{SB}-H) and Ser172 hydroxyl is blue contoured at 2 σ level. (**E**) Superposition of PLP-Gly with PLP internal aldimine (PDB ID 9BPE). Rotations of Schiff bases relative to pyridine rings are shown as dihedral angles (orange sticks). (**F**) Position of Lys226. Brown dashed lines depict N-H···π interactions, the purple double arrow indicates no H bond from Lys226 to Thr223. (**G**) Binding of the pterin moiety of 5MTHF. (**H**) The pocket surrounding 5-methyl group of 5-MTHF. Conventional H bonds are shown as black dashes, unconventional C-H···O H bonds are depicted as orange dashes. * denotes the residue belongs to protomer A. Distances are in Angstroms. Figure is generated using PyMol (Schrödinger, Inc.).

Figure 3. Neutron scattering length density map and interactions for the apo-active site in protomer A of *Tth*SHMT/PLP-Gly/5MTHF complex. (**A**) The $2F_O$-F_C map shown as light brown mesh is contoured at 1 σ level. Density for SO_4^{2-} interacting with Arg358* is omitted for clarity. (**B**) H bonding interactions. Asterisks denote the residue belongs to protomer A. Conventional H bonds are shown as black dashes. Distances are in Angstroms. PDB ID 9O5G. Figure is generated using PyMol (Schrödinger, Inc.).

Figure 4. The organization of the active site in protomer B of *Tth*SHMT/THF complex and comparison with *Tth*SHMT/PLP-Gly/5MTHF and *Tth*SHMT/FA complexes. (**A**) The $2F_O$-F_C electron density map is shown as violet mesh contoured at 1.5 σ level. (**B**) H bonding interactions between PLP, SO_4^{2-}, THF and the active site residues (PDB ID 9O50). (**C**) Superposition of protomers B from *Tth*SHMT/PLP-Gly/5MTHF (grey carbon atoms and cartoon, PDB ID 9O5G), *Tth*SHMT/FA (orange carbon atoms and cartoon, PDB ID 9BPE), and *Tth*SHMT/THF (yellow carbon atoms, green cartoon, PDB ID 9O50) structures. Distances are in Angstroms. Figure is generated using PyMol (Schrödinger, Inc.).