Crystal Structure of a Distinct Type of Methylenetetrahydrofolate Reductase from *Sphingobium lignivorans* SYK-6

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in one-carbon metabolism. We investigated an unusual MTHFR from *Sphingobium lignivorans* SYK-6 (S6MTHFR) that catalyzes the reverse reaction of typical MTHFRs. Our biochemical assays provided the first direct evidence that S6MTHFR catalyzes the oxidation of methyltetrahydrofolate (CH_3-THF). To understand the mechanism of the reverse catalytic reaction, we determined the crystal structures of S6MTHFR. The structures explained why the enzyme cannot catalyze the reduction of methylenetetrahydrofolate (CH_2-THF). Database search based on the structural information of S6MTHFR established a new Type 4 MTHFR family that couples with demethylases.

One-carbon (1C) metabolism is a set of fundamental chemical reactions essential for all life, underpinning the synthesis of nucleic acids, amino acids, and other critical biomolecules. MTHFR is one of the key enzymes of the 1C metabolism, and it catalyzes the reduction of CH_2-THF to CH_3-THF. This reaction is a crucial step in the folate cycle, ultimately providing the methyl group necessary for synthesizing methionine, an essential amino acid. However, the bacterium *Sphingobium lignivorans* SYK-6, known for its ability to break down lignin-derived aromatic compounds, presented a metabolic puzzle. It possesses an MTHFR enzyme (S6MTHFR) that was predicted to catalyze the reverse reaction, the oxidation of CH_3-THF to CH_2-THF. This distinctive characteristic was suggested by SYK-6's unique auxotrophy for methionine and by metabolic flux analyses [1, 2], but direct biochemical evidence was lacking, and the molecular basis for this reversed reactivity was unknown. Our study aimed to definitively characterize S6MTHFR, elucidate the structural basis for its unique function, and explore its broader significance in the microbial world.

Our enzymatic assays provided the first direct evidence of S6MTHFR's unique catalytic reaction. We demonstrated conclusively that S6MTHFR efficiently catalyzes the oxidation of CH_3-THF to CH_2-THF, with a specific activity significantly higher than that of the conventional *E. coli* MTHFR (EcMTHFR) [3]. Conversely, S6MTHFR showed almost no activity with NADH, the typical electron donor for conventional MTHFRs to reduce CH_2-THF. This confirmed that S6MTHFR catalyzes the reaction in the reverse direction compared to typical MTHFRs [3]. To uncover the molecular mechanism underlying this reversed catalysis, we determined crystal structures of S6MTHFR, the substrate-free and -complex forms at 1.50 Å and 1.85 Å resolution, respectively (Fig. 1) [4]. The diffraction data for the substrate-free and the CH_3-THF complex structures were collected at beamline BL-1A of the Photon Factory (KEK) and at beamline X06DA of the Swiss Light Source (SLS), respectively. These crystal structures provided unprecedented insight. S6MTHFR forces the substrate, CH_3-THF, into a compact, folded conformation within its active site unlike typical MTHFRs, which bind

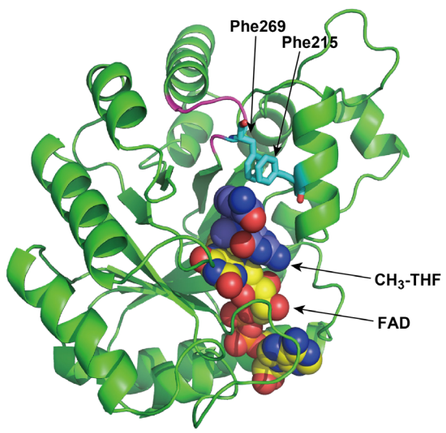

Figure 1: Overall structure of S6MTHFR-CH_3-THF complex. The carbon atoms of S6MTHFR, FAD, and CH_3-THF are shown in green, yellow, and violet. The loop β8-α11 is shown in purple.

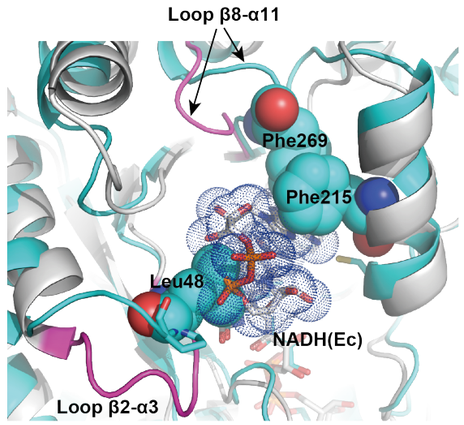

Figure 2: NADH cannot bind to the active site of S6MTHFR. EcMTHFR (white) and S6MTFHR (cyan) are superimposed. Phe215 and Phe269 of S6MTHFR hamper NADH binding. Loops β2-α3 and β8-α11 of EcMTHFR are shown in purple.

their substrate in an extended conformation. This folded structure is stabilized by interactions with specific amino acid residues, including Phe215 and Phe269, and appears to create a geometry optimal for the oxidation reaction of CH_3-THF. Furthermore, the structures explained why S6MTHFR cannot use NADH, which is required for the reduction of CH_2-THF. Loops β2-α3 (residues 46-52) and β8-α11 (residues 268-272) adopt conformations different from typical MTHFRs, which create steric clashes, physically blocking NADH from binding to the active site (Fig. 2). To validate our structural findings, we performed site-directed mutagenesis. We replaced these key loop residues with those of EcMTHFR. The mutant with the EcMTHFR amino acid sequences at the key loops showed an NADH-dependent CH_2-THF reducing activity. This experiment clearly shows that the amino acid sequence of the key loops is critical for the reverse catalytic reaction of S6MTHFR.

Next, we asked if there are homologs of S6MTHFR, which catalyzes the reverse catalytic reaction of typical MTHFRs, based on the structural features of the key loops. A database search revealed many homologs of S6MTHFR, which share the amino acid sequences of the key loops with S6MTHFR. Interestingly, bacteria harboring S6MTHFR homologous enzymes also possess THF-dependent demethylases – enzymes that produce CH_3-THF. Phylogenetic analysis showed that these S6MTHFR homologs form a distinct cluster from existing types 1, 2, and 3 of MTFHRs. Thus, we have classified S6MTHFR homologs as a Type 4 MTHFR family [3]. These findings establish a distinct type of 1C metabolism. In this pathway, bacteria use THF-dependent demethylases, LigM, to capture a one-carbon unit from environmental sources (like the methoxy groups in lignin derivatives), producing CH_3-THF [5, 6]. The Type 4 MTHFR enzyme then oxidizes this CH_3-THF to CH_2-THF, feeding a carbon unit into the central folate cycle for the synthesis of purines and other essential molecules. This coupled system may function as an "environmental monitor," allowing the cell to regulate methionine levels and overall growth based on the availability of specific carbon sources.

In summary, our research, enabled by the high-resolution structural data obtained at the Photon Factory, has not only uncovered the unique properties of S6MTHFR but has established a distinct family of MTHFRs.

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