Mutagenesis of Folylpolyglutamate Synthetase Indicates That Dihydropteroate and Tetrahydrofolate Bind to the Same Site†

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ABSTRACT: The folylpolyglutamate synthetase (FPGS) enzyme of *Escherichia coli* differs from that of *Lactobacillus casei* in having dihydrofolate synthetase activity, which catalyzes the production of dihydrofolate from dihydropteroate. The present study undertook mutagenesis to identify structural elements that are directly responsible for the functional differences between the two enzymes. The amino terminal domain (residues 1−287) of the *E. coli* FPGS was found to bind tetrahydrofolate and dihydropteroate with the same affinity as the intact enzyme. The domain-swap chimera proteins between the *E. coli* and the *L. casei* enzymes possess both folate or pteroate binding properties and enzymatic activities of their amino terminal portion, suggesting that the N-terminal domain determines the folate substrate specificity. Recent structural studies have identified two unique folate binding sites, the omega loop in *L. casei* FPGS and the dihydropteroate binding loop in the *E. coli* enzyme. Mutants with swapped omega loops retained the activities and folate or pteroate binding properties of the rest of the enzyme. Mutating *L. casei* FPGS to contain an *E. coli* FPGS dihydropteroate binding loop did not alter its substrate specificity to using dihydropteroate as a substrate. The mutant D154A, a residue specific for the dihydropteroate binding site in *E. coli* FPGS, and D151A, the corresponding mutant in the *L. casei* enzyme, were both defective in using tetrahydrofolate as their substrate, suggesting that the binding site corresponding to the *E. coli* pteroate binding site is also the tetrahydrofolate binding site for both enzymes. Tetrahydrofolate diglutamate was a slightly less effective substrate than the monoglutamate with the wild-type enzyme but was a 40-fold more effective substrate with the D151A mutant. This suggests that the 5,10-methylenetetrahydrofolate binding site identified in the *L. casei* ternary structure may bind diglutamate and polyglutamate folate derivatives.

Folylpolyglutamate synthetase (FPGS,[1] EC 6.3.2.17) catalyzes the MgATP-dependent addition of l-glutamate to folate compounds. In organisms that require exogenous folates for growth, such as *Lactobacillus casei*, *Streptococcus faecalis*, and mammals, FPGS serves to synthesize a highly anionic polyglutamate chain of four residues or longer on folate substrates to retard folate transport through the cell membrane (*1*, *2*). This is the mechanism thought to be responsible for the accumulation and maintenance of cellular folate pools. Many bacteria, such as *Escherichia coli* and *Corynebacterium* sp., can synthesize folate de novo. The FPGS enzymes from these bacteria have dihydrofolate synthetase (DHFS) activity, which catalyzes the addition of L-glutamate to dihydropteroate to form dihydrofolate. This enzyme is part of the de novo folate biosynthetic pathway. A lack of DHFS and other folate biosynthetic enzymes in *Lactobacillus* causes its nutritional requirement for folate, first discovered by Snell and Peterson (*3*). This discovery led to the use of *Lactobacillus* in folate microbiological assays, which facilitated the purification and identification of folic acid as a vitamin and is still an assay that is used today. Since *E. coli* does not have specific transporters for folates, FPGS-DHFS is essential (*4*). In yeast, there are two separate enzymes for FPGS and DHFS activities, and DHFS is essential in the absence of exogenous folate (*5*, *6*). Functional differences of FPGS enzymes in bacteria and mammals may be explored for rational drug design. A specific inhibitor of DHFS activity of the bacteria enzyme may kill pathogenic bacteria without harming the host, and since mammalian cells lack DHFS, such an inhibitor could be an effective antimicrobial drug. Thus, a detailed understanding of the structural basis for the differential binding of folate compounds by *L. casei* FPGS and *E. coli* FPGS-DHFS may be useful in elucidation of the mechanism of the enzymes and validation of the potential of FPGS as a drug target.

L. casei FPGS and *E. coli* FPGS-DHFS differ from one another in their preference of folate substrates and affinity for ATP. *L. casei* FPGS prefers mTHF as its folate substrate and synthesizes predominantly octa- and nonaglutamates in vivo when grown with low folate concentrations (*7*). ATP

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[1] Abbreviations: FPGS, folylpoly-γ-glutamate synthetase; MurD, UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase; DHFS, dihydrofolate synthetase; $H_4PteGlu_2$, tetrahydrofolate diglutamate; ACP4, β,γ-methylene adenosine tetraphosphate; THF, tetrahydrofolate; mTHF, 5,10-methylenetetrahydrofolate; DHP, dihydropteroate; DMSO, dimethylsulfoxide; K_d, dissociation constant; K_m, Michaelis constant; WT, wild-type.

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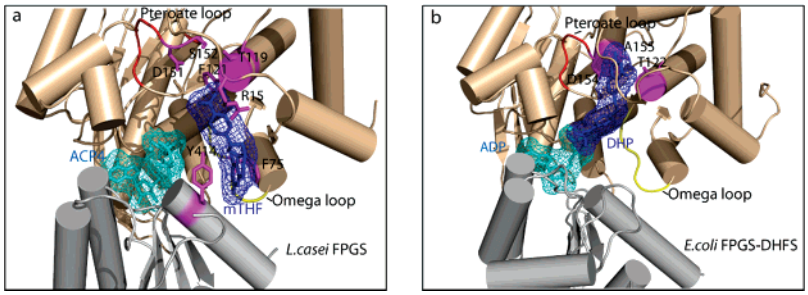

FIGURE 1: Structural diagrams of *L. casei* FPGS (a) with mTHF (minus its glutamate residue) bound at the mTHF binding site and *E. coli* FPGS (b) bound in the DHP binding site. The N- and C-terminal domains are in brown and gray, respectively. Both DHP and mTHF are shown in dark blue in their respective diagrams. The omega loop is shown in yellow in both diagrams, and the residues corresponding to the pteroate binding loop of *E. coli* FPGS are in red. The nucleotides ADP in panel a and ACP4 in panel b are light blue in their binding pocket. The location of selected residues that were mutated in this study are indicated. The numbering of the residues is the numbering system for their respective enzymes.

is required for *L. casei* FPGS to function, but its K_m value is high (3 mM) as compared to enzymes from other sources (10−70 μM) (*8*, *9*). The K_m value for ATP of *E. coli* FPGS is 50 μM. *E. coli* FPGS-DHFS has a broader folate specificity than *L. casei* FPGS. It prefers 10-fomyl-THF as its monoglutamate substrate and mTHF as its diglutamate substrate but will also efficiently use THF (*10*, *11*). DHP is the substrate associated with the *E. coli* DHFS activity that has the highest affinity for the enzyme. Folate triglutamates are the predominant products of *E. coli* FPGS.

The crystal structure of *L. casei* FPGS (ref *12*, RCSB Protein Data Bank accession no. 1JBW) reveals that this enzyme has a modular architecture consisting of N- and C-terminal domains connected by a linker loop. The structure of the ternary complex of *L. casei* FPGS with β,γ-methyleneadenosine tetraphosphate and mTHF shows that the mTHF binding site is located in a cleft between the N- and the C-terminal domains adjacent to the omega loop (residues 72−82, see Figure 1) (*13*). However, the binding site for mTHF monoglutamate is too far from the ATP binding site to allow catalysis. A recent computational study (*14*) identified a binding pocket for folate distinct from that in our crystal structure, which accommodates monoglutamate substrates and has more favorable free energies of binding for most substrates. It was suggested that the site observed in the ternary complex structure may be utilized by some folate polyglutamate substrates. The recent crystal structure of the *E. coli* FPGS shows that DHP is bound essentially in this predicted pocket (ref *15*, RCSB Protein Data Bank accession nos. 1W7K and 1W78). Mathieu et al. (*15*) suggested that there may be distinct sites in FPGS for DHP and THF binding. The present study undertakes mutagenesis to identify structural elements that are responsible for the unique folate binding characteristics of *E. coli* FPGS-DHFS and *L. casei* FPGS and to shed light on the catalytic mechanisms of FPGS versus FPGS-DHFS. We made a series of chimeric constructs of *E. coli* FPGS-DHFS and *L. casei* FPGS that showed that the N-terminal domain is sufficient for binding both THF and DHP, while ATP binding needs the intact N- and C-terminal structure. We constructed omega loop exchange mutants between *E. coli* and *L. casei* FPGS to determine the effect of that structure in the folate or pteroate substrate specificity of enzymes. We also mutagenized individual residues to determine as to whether the new pteroate binding site described in the *E. coli* crystal structure is also utilized by THF substrates and as to whether the corresponding site in *L. casei* FPGS, described in Tan and Carlson's study (*14*), is utilized by the enzyme. Aspartate 151 in *L. casei* FPGS and the corresponding aspartate 154 in *E. coli* FPGS are predicted to be involved in folate binding to the new pteroate binding site but not the mTHF binding site identified in the *L. casei* ternary complex crystal structure. Mutation of these residues substantially decreased folate and pteroate binding and FPGS activity in both enzymes, suggesting that the new pteroate binding site is the primary site for pteroate and folate monoglutamate binding. The greater activity of these mutants with tetrahydrofolate diglutamate substrates suggests that the mTHF binding site found in *L. casei* FPGS may be utilized by folate diglutamate and polyglutamate substrates.

MATERIALS AND METHODS

Materials. (6*RS*)-5,6,7,8-THF and 7,8-dihydropteroate were from Schircks Laboratories. mTHF was prepared from THF by the addition of formaldehyde in excess to the assay mix. THF di- and triglutamates were prepared by the reduction of folic acid di- and triglutamates with sodium dithionite and stored at −70 °C as described previously (*16*) followed by reduction of the dihydro forms using dihydrofolate reductase (*17*).

Construction of Plasmids. Oligonucleotide primers used for constructing various mutants are shown in Table S1. Mutagenesis was performed on the *L. casei* FPGS or *E. coli* FPGS genes using the construct in the expression plasmid pCYB1(NEB) as the template. The constructs are shown in Figure 2. For the construct E_{1-288}, an *Nde*I site was introduced into the 5′ end of the forward primer, and a *Sap*1 site was introduced into the 5′ end of the reverse primer. The PCR fragment was cut with restriction enzymes *Nde*I and *Sap*I and inserted into the pCYB1 vector prepared by digestion with the same enzymes. For the chimera proteins $L_{1-298}/E_{288-428}$, $E_{1-287}/L_{299-428}$, $L_{1-154}/E_{158-422}$, and $E_{1-157}/L_{155-428}$, PCR (30 cycles) was used to generate two DNA fragments, one from *L. casei* FPGS and the other from *E. coli* FPGS-DHFS. An *Nde*I and a *Sap*I site was introduced into the forward and reverse primers, respectively, of the DNA fragment encoding the amino terminus of the chimera protein. A *Sap*I and an *Asp*718 site was introduced into the forward and reverse primers, respectively, of the DNA fragment for the carboxyl terminus. The two fragments were cut with the appropriate enzymes and ligated with the pCYB1 vector that was linearized with *Nde*I and *Asp*718. The L_{1-71}/

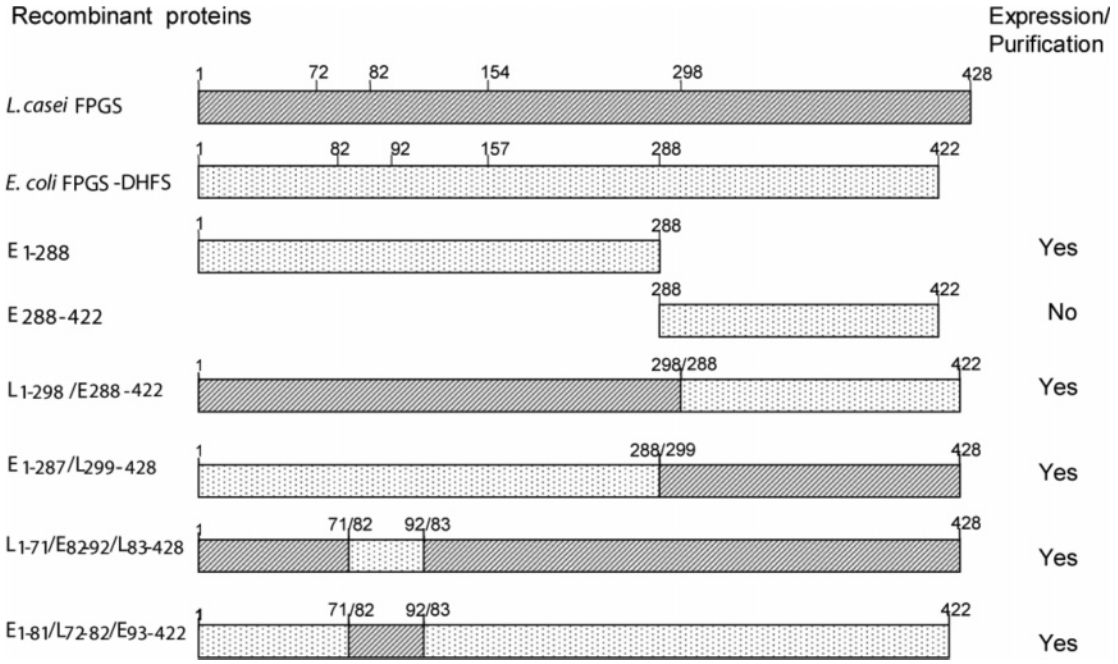

FIGURE 2: FPGS-DHFS mutants. The column on the left is a schematic representation of the mutants showing pertinent regions of the protein. The column on the right indicates the expression levels of the recombinant protein.

E_{82-92}/L_{83-425} and $E_{1-81}/L_{72-82}/E_{93-422}$ mutants were generated using primer pairs that contained the entire omega loop. The oligonucleotide primer pairs were 5′ phosphorylated and annealed, leaving three nucleotide overhangs at their 5′ ends. A linear PCR product synthesized outward from primers bordering the omega loop including the enzyme coding sequence and the pCYB1 vector was generated by two primers containing *Sap*1 restriction sites at their 5′ ends. This PCR product was digested with *Sap*1, producing 5′ overhangs complementary to the omega loop fragment overhangs, and the two were ligated.

The point mutants were constructed using the Gene Editor (Promega) mutagenesis kit and following the manufacturer's instructions. The genes were mutagenized in pBluescript plasmids and subsequently subcloned into pCYB1 for expression. All constructs were confirmed by DNA sequencing.

Expression and Purification of Mutant Proteins. The plasmid encoding a mutant protein was transformed into *E. coli* strain BL21 for expression. FPGS mutants were expressed as fusion proteins using the IMPACT system from NEB. The cultures were induced with 1 mM IPTG at 30 °C for 6 h. The crude extracts were loaded on a chitin column, and the fusion proteins were cleaved with 50 mM DTT in 10 mM Tris HCl, pH 7.5, 1 M NaCl for 24 h at room temperature (see Figures S1 and S2). The eluted proteins were dialyzed against 10 mM Tris HCl, pH 7.5, 200 mM KCl and stored at −70 °C with 20% DMSO. The protein concentration was determined by a Bradford assay (Bio-Rad).

Enzymatic Assay. Enzyme activities were measured by the incorporation of [^{3}H]-glutamate into 5,10-methylene-tetrahydrofolate, as described by Shane (*18*). A standard *L. casei* FPGS assay mix consisted of 100 mM Tris (pH 9.75), 50 mM glycine, 200 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 10% DMSO, 5 mM ATP, 250 μM L-glutamate, 1.25 μCi [^{3}H]-glutamate, 100 μM THF, 12 mM formaldehyde, and 5−10 μg of pure enzyme. For the *E. coli* FPGS-DHFS assay, either THF or DHP was used as the substrate instead of mTHF.

Radioligand Binding (Equilibrium Dialysis). Microdialysis cells with a dialysis membrane (MW cutoff of 12 kDa) were used. One chamber was filled with 120 μL of FPGS protein (1.5 mg/mL) in 10 mM Tris HCl, pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, and 10% DMSO (P-side). The other side was filled with various concentrations of [γ-^{32}P]-ATP in 120 μL of the same buffer as the P-side. The equilibrium was allowed to proceed for 48 h at 4 °C, and the amount of labeled substrate in both sides was measured.

Fluorescence Titration and Fluorescence Quenching. The direct binding of mTHF to FPGS proteins was characterized via intrinsic fluorescence quenching using an AVIV instruments (model ATF105) equipped with excitation intensity correction. Each 0.1 mL sample contained 10 μM protein in 10 mM Tris HCl (pH 7.5), 200 mM KCl, and 10 mM $MgCl_2$. The quenched tryptophan fluorescence signal was measured at increasing mTHF concentrations. The protein samples were excited at 295 nm and emitted at 340 nm with the excitation and emission band-pass set at 3 nm. The K_d values were determined by fitting the fluorescence data to a hyperbolic regression analysis program HYPER (version 1.0).

Constructions of Chimera Proteins. Domain deletion mutants of the *E. coli* enzyme, having the N-terminal domain (residues 1−295, E_{1-295}) and the C-terminal domain (residues 296−425, $E_{296-422}$) expressed separately, were constructed. The N-terminal domain formed a folded structure that migrated on SDS-PAGE around 32 kDa (not shown). We were not able to purify the C-terminal domain.

RESULTS

Chimera proteins have been shown to be useful as tools for understanding structure−function relationships (*19*). To better understand the difference between the *L. casei* and *E. coli* FPGS enzymes, the domain-exchange chimera mutants,

Table 1: Binding Constant K_d for Methylene THF and DHP

proteins	K_d for methylene THF (μM)	K_d for DHP (μM)
L. casei WT	13	NA[a]
E. coli WT	23	6.3
E_{1-288}	98	12
$L_{1-298}/E_{288-422}$	104	NA
$E_{1-287}/L_{299-428}$	107	5.5
$L_{1-71}/E_{82-92}/L_{83-428}$	23	203
$E_{1-81}/L_{72-82}/E_{93-422}$	31	10

[a] No activity detected.

$L_{1-298}/E_{288-422}$ and $E_{1-287}/L_{299-428}$, and the omega loop exchange mutants, $L_{1-71}/E_{82-92}/L_{83-428}$ and $E_{1-81}/L_{72-82}/E_{93-422}$, were generated, expressed, and purified (see Figure 2). The purified mutant proteins were examined for their folding properties, enzyme activity, and the ability to bind ATP and folate. CD spectrometry was used to scan all the purified proteins to ensure that the proteins folded normally. To evaluate the ligand binding affinity, equilibrium dialysis using [γ-^{32}P]-ATP was performed to obtain the binding constants (K_d) for ATP, and fluorescence titration was used for deducing the DHP binding constants.

N-Terminal Domain of E. coli FPGS Is Sufficient for Binding THF and Pteroate. To test the function of the N-terminal domain of FPGS on folate binding, we cloned the N-terminal domains of *L. casei* FPGS and *E. coli* FPGS-DHFS. Only the N-terminal domain of the *E. coli* enzyme but not of *L. casei* enzyme could be expressed and purified. Although no enzyme activity or ATP binding could be detected with the N-terminal domain mutant, fluorescence titration studies using DHP showed that it was able to bind DHP with a 2-fold higher K_d value than the WT enzyme (12 μM vs 6.3 μM) (Table 1and Figure 4a). mTHF binding was studied using intrinsic fluorescence quenching. The N-terminal domain was able to bind mTHF with a K_d value 4-fold higher than the WT protein (98 μM vs 23 μM) (Table 1 and Figure 4a). This demonstrates that the N-terminal domain alone is sufficient to bind both THF and DHP substrates but not for ATP binding. Since we have previously shown that E143 in the N-terminal domain is required for ATP binding (*20*), we conclude that ATP requires both the N- and the C-terminal domains for binding.

Domain Switch Mutants. $L_{1-298}/E_{288-422}$ contains the N-terminal domain of *L. casei* FPGS (residues 1−298) and the C-terminal domain (residues 288−422) of *E. coli* FPGS; $E_{1-287}/L_{299-428}$ contains the N-terminal domain of *E. coli* FPGS (residues 1−287) and the C-terminal domain (residues 299−428) of *L. casei* FPGS. The conserved linker loop was chosen as the switch site. The chimera and WT proteins had a similar mobility on SDS-PAGE with molecular weights of about 43 kDa. Enzyme activity of both chimera proteins was examined using mTHF to measure FPGS activity and DHP to measure DHFS activity. As shown in Table 2, $L_{1-298}/E_{288-422}$ had 11% WT *L. casei* FPGS activity but no DHFS activity. $E_{1-287}/L_{299-428}$ had no activity, although the circular dichroism spectrum shows that the global folding of the protein is not affected. The K_m value for ATP of $L_{1-298}/E_{288-422}$ was 5.7 mM, which is 1.7-fold higher than that of the *L. casei* WT enzyme and 100-fold higher than that of the *E. coli* WT enzyme (Table 2). The K_m value for glutamate was 292 μM, which is not very different from the K_m value of either the *L. casei* or the *E. coli* WT enzymes, indicating that the glutamate binding is not affected. The K_m value for mTHF (590 μM) was increased 18-fold as compared to the *L. casei* WT and 12-fold as compared to the *E. coli* WT.

Next, we measured the direct binding of DHP and mTHF by the chimeric enzymes. Figure 4b shows the quenched tryptophan fluorescence intensity against the increased concentration of mTHF. $L_{1-298}/E_{288-422}$ and $E_{1-287}/L_{299-428}$ both bound mTHF. The K_d values for mTHF of $L_{1-298}/E_{288-422}$ and $E_{1-287}/L_{299-428}$ were 104 and 107 μM, respectively, which are 5-fold higher than for their WT proteins (Table 1). The $E_{1-287}/L_{299-428}$ mutant had no enzyme activity, showing that folate binding is not sufficient for FPGS function. Figure 3b shows the change in the intensity of DHP fluorescence against the increasing concentration of DHP. While the WT *L. casei* FPGS and $L_{1-298}/E_{288-422}$ mutant did not bind DHP, $E_{1-287}/L_{299-428}$ bound DHP with a similar affinity as *E. coli* FPGS-DHFS. This suggests that the C-terminal domain mutation did not affect the ability of $E_{1-287}/L_{299-428}$ to bind DHP but did affect enzyme function. The C-terminal domain per se did not affect the pteroate/folate substrate specificity of the FPGS enzymes; therefore, $L_{1-298}/E_{288-422}$ had the properties of *L. casei* FPGS, and $E_{1-287}/L_{299-428}$ had the properties of *E. coli* FPGS. We conclude from the previous experiments that the N-terminal domain alone is able to bind folate and determines both FPGS and DHFS binding specificities.

Equilibrium dialysis was performed on the *E. coli* WT enzyme and the mutant $E_{1-287}/L_{299-428}$ using [γ-^{32}P]-ATP as the substrate in the presence of 100 μM DHP. ATP binding was not detected in $E_{1-287}/L_{299-428}$ within the tested ATP concentration (0−100 μM), whereas the WT bound ATP with a K_d value of 30 μM (not shown). This suggests that a loss of activity of the mutant $E_{1-287}/L_{299-428}$ is due to defective ATP binding caused by the mutation.

Omega Loop Exchange Chimeras. The *L. casei* FPGS crystal structure shows the substrate, mTHF, bound within the cleft formed by the omega loop from the N-terminal domain and elements of the C-terminal domain including Y414 (Figure 1a). There is no apparent contact with other parts of the structure (*13*). Therefore, it was of interest to know as to whether the omega loop determines the substrate specificity for folate binding. We constructed a pair of chimeras with only the omega loop (73−81) switched between the two enzymes.

Both the omega exchange mutants were found to be active. When using mTHF as a substrate to measure their FPGS activity, $L_{1-71}/E_{82-92}/L_{83-428}$ showed a 13-fold decrease, and $E_{1-81}/L_{72-82}/E_{93-422}$ revealed a 96-fold decrease in specific activity as compared to their WT counterparts (Table 2). In terms of DHFS activity, $L_{1-71}/E_{82-92}/L_{83-428}$ showed no activity, just like the *L. casei* WT enzyme, while $E_{1-81}/L_{72-82}/E_{93-422}$ was still active but revealed a 11-fold decrease in enzymatic activity as compared to the *E. coli* WT enzyme. The K_m value for mTHF of $L_{1-71}/E_{82-92}/L_{83-428}$ and $E_{1-81}/L_{72-82}/E_{93-422}$ showed 4- and 2-fold increases, respectively, as compared to their WT counterparts. However, the K_m value for DHP of $E_{1-81}/L_{72-82}/E_{93-422}$ showed a 20-fold increase as compared to the WT *E. coli* enzyme.

The two mutants were also analyzed for the direct binding of mTHF and DHP. mTHF bound to $L_{1-71}/E_{82-92}/L_{83-428}$ with a K_d value of 23 μM and to $E_{1-81}/L_{72-82}/E_{93-422}$ with a K_d

Table 2: Enzyme Activities and Kinetic Constants of Chimera Proteins

proteins	specific activity with mTHF (nmol/mg/h)	specific activity with DHP (nmol/mg/h)	K_m[a] for ATP with mTHF (μM)	K_m[a] for glutamate with mTHF (μM)	K_m[a] for mTHF (μM)	K_m[a] for DHP (μM)
L. casei WT	11800	NA[b]	3400	470	32	ND[c]
E. coli WT	13200	4100	54	300	50	6.3
E_{1-288}	NA	NA	ND	ND	ND	ND
$L_{1-298}/E_{288-422}$	1300	NA	5700	292	590	ND
$E_{1-287}/L_{299-428}$	NA	NA	ND	ND	ND	ND
$L_{1-71}/E_{82-92}/L_{83-428}$	912	NA	ND	ND	118	ND
$E_{1-81}/L_{72-82}/E_{93-422}$	138	376	ND	ND	71	134

[a] V_{max} and K_m values for the WT enzymes are true values obtained in previous studies (*7*, *9*) and verified in this study. The values are apparent V_{max} and K_m values with some mutants if the apparent K_m values are much higher than the WT enzyme because full saturation could not be achieved due to the insensitivity of the assay at high glutamate concentrations and substrate inhibition at high ATP and THF concentrations. [b] No activity detected. If the activity detected is <0.1% of the specific activity of the WT enzyme, it is considered to be of no activity. If the assay with the WT is 50 000 dpm, this is a value of 50 dpm at equal protein concentrations, where the background is about 100 dpm. [c] Not determined. The K_m value for ATP and glutamate of the chimera proteins $L_{1-71}/E_{82-92}/L_{83-428}$ and $E_{1-81}/L_{72-82}/E_{93-422}$ were the only constants not determined in enzymes that had activity.

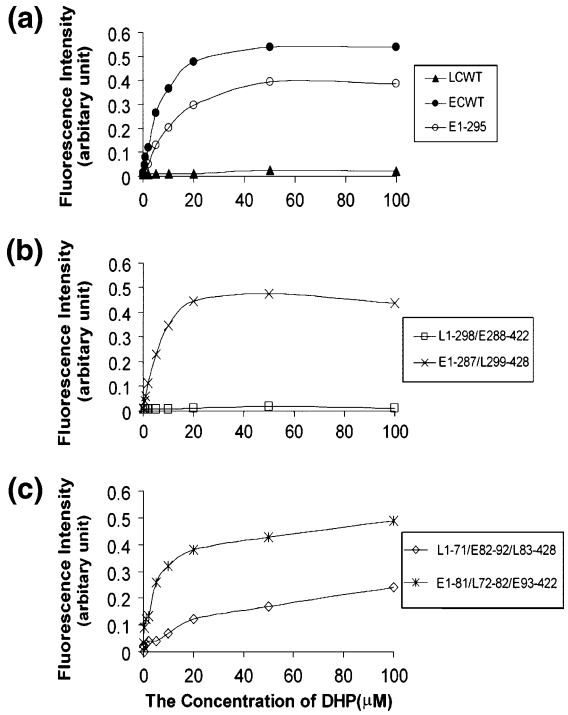

FIGURE 3: DHP fluorescence signals measured by the difference in fluorescence intensity in the presence vs the absence of the proteins plotted against the increased concentration of DHP over a range of concentrations from 0 to 100 μM. (a) *L. casei* WT protein, *E. coli* WT protein, and E_{1-288}. (b) $L_{1-298}/E_{288-422}$ and $E_{1-287}/L_{299-428}$. (c) $L_{1-71}/E_{82-92}/L_{83-428}$ and $E_{1-81}/L_{72-82}/E_{93-422}$.

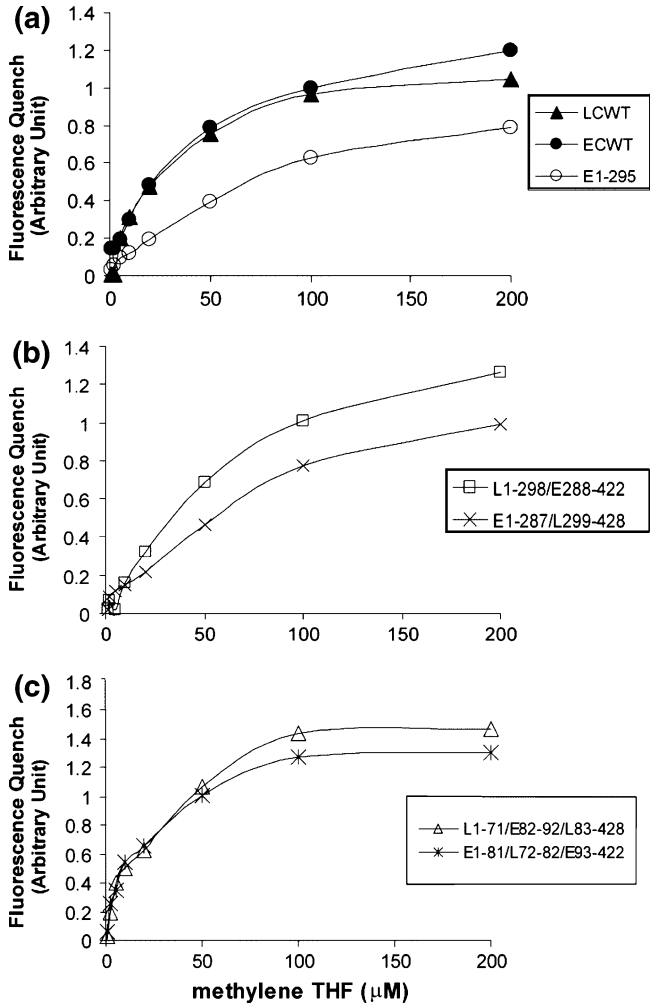

FIGURE 4: Tryptophan fluorescence quenching signals of the mutant proteins in the presence of 5,10-methylene-THF over a range of concentrations from 0 to 200 μM. (a) *L. casei* WT protein, *E. coli* WT protein, and E_{1-288}. (b) $L_{1-298}/E_{288-422}$ and $E_{1-287}/L_{299-428}$. (c) $L_{1-71}/E_{82-92}/L_{83-428}$ and $E_{1-81}/L_{72-82}/E_{93-422}$.

value of 31 μM (Table 1). Both had a similar binding affinity as their WT counterparts. The K_d value for DHP of $E_{1-81}/L_{72-82}/E_{93-422}$ was 10 μM, which is not much different from the K_d value of the WT *E. coli* enzyme (6.3 μM). In contrast, $L_{1-71}/E_{82-92}/L_{83-428}$ had weak binding with DHP, resulting in a K_d value of 203 μM. The results indicated that the omega loop switch did not result in a complete switch in enzyme activity. $L_{1-71}/E_{82-92}/L_{83-428}$ was similar to *L. casei* FPGS, while $E_{1-81}/L_{72-82}/E_{93-422}$ behaved like the *E. coli* enzyme. It shows that switching the omega loop does not switch the folate substrate specificity of FPGS. An intact three-dimensional environment is required for the determination of FPGS/DHFS folate substrate specificity. However, the binding of DHP to $L_{1-71}/E_{82-92}/L_{83-428}$ shows that the omega loop does contribute to folate binding and folate substrate specificity.

Mutations at the Pteroate Binding Site. A crystal structure of *E. coli* FPGS identified a pteroate binding site in the enzyme (*15*). The binding pocket to which the pteroate bound also exists in *L. casei* FPGS (see the pteroate binding loop

Table 3: Kinetic Properties of the *L. casei* Mutant Proteins

protein	folate substrate	mTHF K_m[a]	ATP K_m[a] (mM)	glutamate K_m[a] (μM)	V_{max}[a] (μmol/h/mg)
WT	mTHF	32 μM	3.4	470	19
	mTHFGlu2	27 μM	ND[b]	ND	14
F75A	mTHF	230 μM	0.18	53	1.0
D151A	mTHF	110 μM	3.4	ND	0.3
	mTHFGlu2	10 μM	ND	ND	1.2
R15E	mTHF	84 μM	ND	ND	17
	mTHFGlu2	204 μM	ND	ND	22
LC5DEL	mTHF	90 μM	ND	ND	3.6
	mTHFGlu2	10 μM	ND	ND	2.9
S152W	mTHF	116 μM	0.44	ND	1.1
S152A	mTHF	34 μM	ND	ND	1.1
T119W	mTHF	3.8 mM	ND	ND	16.
F121A	mTHF	1.5 mM	ND	ND	6.4
Y414A	mTHF	48 μM	0.23	2700	0.1

[a] V_{max} and K_m values for the WT enzymes are true values obtained in previous studies (*7*, *9*) and verified in this study. The values are apparent V_{max} and K_m values with some mutants if the apparent K_m values are much higher than the WT enzyme because full saturation could not be achieved due to the insensitivity of the assay at high glutamate concentrations and substrate inhibition at high ATP and THF concentrations. [b] Not determined.

Figure 1), and it is distinct from the pocket that bound the pteroate moiety of mTHF in the mTHF-ACP4 ternary structure with *L. casei* FPGS (*13*). The region that interacts with the glutamate moiety of folates bound in the two pockets overlaps in modeling studies, including such conserved residues as S73, R82, T119, and F121 in *L. casei* FPGS and the corresponding residues S83, R92, T122, and F124 in *E. coli* FPGS (*14*). D154 in *E. coli* FPGS is a key residue that interacts with the pterin group of DHP and is specific for the pteroate binding pocket (*15*). D151, the corresponding residue in *L. casei* FPGS, did not show any interactions in the mTHF-ACP4-*L. casei* FPGS complex structure. The pteroate of mTHF bound in the mTHF-ACP4 pteroate binding pocket interacts with F75 and Y414 of *L. casei* FPGS (Figure 1). To assess the specific role in the folate binding specificity of residues in the identified folate binding site in the crystal structures, we made a series of single mutations at sites including R15, F75, T119, F121, D151, S152, and Y414 in *L. casei* FPGS and T122, D154, and A155 in *E. coli* FPGS.

We constructed a F121A mutant of *L. casei* FPGS, which is predicted to interact with the pteroate moieties of substrates bound at either binding site (Figure 1). This mutant enzyme has a 50-fold increase in the K_m value for mTHF, consistent with it playing an important role in folate binding (Table 3). F75 interacts with the pterin of mTHF in the *L. casei* ternary complex structure. It is, however, part of the omega loop, which is involved in binding both ATP and pteroates and is predicted to interact with the glutamate side chains in both models of pteroate binding (*14*). Thus, mutation of F75 could affect the conformation of the omega loop (*21*), and this residue may not be specific for the mTHF binding pocket. The F75A mutant has a 7.5-fold increase in the K_m value for mTHF and a 20-fold decrease in V_{max}, but it had a significant decrease in K_m for both ATP and glutamate, suggesting that it may be altering the structure of the omega loop. The activity of this mutant is about the same as that of other omega loop mutants, and its K_m value for mTHF is lower than that of the S73A mutant in the omega loop (*22*).

We constructed a D151A mutant FPGS to assess the contribution of the new pteroate binding site to *L. casei* FPGS activity since its analogue in *E. coli* FPGS (D154) interacts directly with DHP (*15*), but D151 is distant from the mTHF binding site in the *L. casei* ternary complex structure (*13*). As compared to the WT enzyme, the D151A mutant had a 63-fold lower V_{max} value and a 3.7-fold higher K_m value with THF as the substrate, resulting in a 230-fold less effective enzyme (Table 3). We also mutated two residues, T119 and S152, on the edge of the pteroate binding pocket to bulky tryptophan or histidine residues, which could potentially block entry into the pocket. The T119W mutant had a 125-fold increase in K_m for mTHF, and the S152W mutant had a 17-fold decrease in V_{max} and a 3.9-fold increase in K_m for mTHF. These data are consistent with the notion that the new pteroate binding pocket identified in *E. coli* FPGS-DHFS also plays an important role in folate binding in the *L. casei* FPGS.

Y414 specifically interacts with mTHF in the *L. casei* ternary complex crystal structure. The Y414A mutant has a V_{max} value that is 128-fold lower than WT, but the K_m value for mTHF increased only 1.5-fold, whereas the K_m value for glutamate increased 6-fold. This agrees with the prediction that Y414 is involved in glutamate binding (*13*, *14*), and it agrees with the finding from the N-terminal and domain switch mutants that residues from the C-terminal domain are not required for pteroate binding. Taken together, these results suggest that the pteroate binding pocket is a functional folate binding site in *L. casei* FPGS and that it is the primary binding site utilized by mTHF.

mTHF Diglutamate May Utilize the L. casei FPGS mTHF Binding Site. mTHF diglutamate had a substrate activity about equal to mTHF monoglutamate with the WT *L. casei* FPGS. With the D151A mutant, mTHF diglutamate had a 4-fold higher V_{max} value than the monoglutamate and an 11-fold lower K_m value (Table 3). This suggests that the mutant protein was less impaired for diglutamate utilization than for monoglutamate utilization. This may reflect the ability of mTHF diglutamate to bind at the ternary complex binding pocket, as shown in Figure 5, and this binding would not be disrupted by the mutation. In contrast, the R15E mutant protein behaved much like the WT enzyme with mTHF diglutamate being about as effective a substrate as monoglutamate, having both a higher K_m and V_{max} value than the monoglutamate. R15 is a residue that lines the active site of *L. casei* FPGS but interacts with the pteroate substrate with its peptide backbone moieties rather than its side chain. It has been suggested to interact with the carbonyl residues of polyglutamate substrates (*14*).

We mutated D154 of *E. coli* FPGS, a residue that interacts directly with DHP and that is at the corresponding site to D151 in the *L. casei* enzyme. The mutant was severely impaired in its activity with THF as the substrate, even more so than with DHP. Both substrates had large increases in K_m, but the V_{max} value with THF was 23-fold lower than WT, whereas DHP had a V_{max} value 2.5-fold lower than the WT enzyme (Table 4). As with *L. casei* FPGS, THF diglutamate was a better substrate with the D154A mutant than the monoglutamate. Its V_{max} value was 10-fold higher than monoglutamate and similar to the V_{max} value of the WT enzyme. However, the K_m value of the diglutamate to the mutant enzyme (300 μM) was similar to that of the

Table 4: Kinetic Properties of the *E. coli* Mutant Proteins

protein	pteroate substrate	THF/DHP K_m[a] (μM)	ATP K_m[a] (μM)	glutamate K_m[a] (μM)	V_{max}[a] μmol/h/mg
D154A	THF	340	230	200	18
	DHP	140	37	3300	5.2
	THFGlu2	300	ND[b]	ND	200
A155W	THF	10	ND	ND	21
	DHP	80	555	ND	15
T122H	THF	400	ND	ND	32
	DHP	980	ND	ND	89
T122W	THF	390	ND	ND	46
	DHP	150	ND	ND	180
A155H	THF	448	76	120	67
	DHP	485	65	170	53
WT	THF	50	54	300	415
	DHP	6.3	7	3900	13
	THFGlu2	1.4	ND	ND	380

[a] V_{max} and K_m values for the WT enzymes are true values obtained in previous studies (*7*, *9*) and verified in this study. The values are apparent V_{max} and K_m values with some mutants if the apparent K_m values are much higher than the WT enzyme because full saturation could not be achieved due to the insensitivity of the assay at high glutamate concentrations and substrate inhibition at high ATP and THF concentrations. [b] Not determined.

monoglutamate (340 μM), whereas the diglutamate had a much lower K_m value with the WT enzyme (1.4 μM). This suggests that the mTHF binding pocket is ineffectively used for the diglutamate substrate by *E. coli* FPGS. Similar to the effect of the corresponding mutations in *L. casei* FPGS, T122W and T122H mutations caused large increases in K_m for both THF and DHP (Table 4), consistent with the bulky residues blocking the pteroate binding site. The A155H mutation had a similar effect, although the A155W mutation did not increase the K_m value for THF.

Mathieu et al. (*15*) refer to residues 25−32 as a dihydropteroate binding loop due to its position proximal to the binding pocket. Early work in our laboratory showed that the first 29 residues of *E. coli* FPGS can be deleted without the loss of either FPGS or DHFS activity (*24*). We did a similar deletion in the corresponding amino terminal region of *L. casei* FPGS in which residues 3−18 were deleted. This mutant protein was expressed and had a FPGS specific activity about 20% WT (Table 4, Lc5′DEL). We investigated as to whether mutating *L. casei* FPGS to have the same sequence as the *E. coli* FPGS DHFS binding loop changed its folate substrate specificity. The mutant enzyme did not acquire DHFS activity but had a normal FPGS activity (not shown).

DISCUSSION

DHFS and FPGS Activities Share the Same Binding Site. *L. casei* FPGS and *E. coli* DHFS/FPGS represent two different classes of folyl-γ-glutamate synthetase. The sequence similarity between *L. casei* FPGS and *E. coli* DHFS/FPGS is about 35% with the conserved regions distributed throughout the entire sequence length. The crystal structure of *L. casei* FPGS in a ternary complex with ACP4 and mTHF has revealed that the mTHF sits adjacent to the omega loop with the pteridine ring interacting with F75 from the omega loop and Y414 from the C-terminal domain (*13*). The *E. coli* FPGS structure showed a different pteroate binding site than the one in *L. casei* FPGS.

Both DHFS and FPGS can catalyze the addition of one or multiple glutamate moieties onto DHP or THF, respectively. The difference between the two classes of substrates is that the former does not contain a glutamate moiety in its chemical structure and that the latter normally consists of one or more glutamate residues linked by γ-peptide bonds. The data in our study do not support this possibility, suggested by Mathieu et al. (*15*), that the *E. coli* enzyme may contain different binding sites for DHP and THF. The domain deletion and chimera mutants as well as previous mutagenesis studies (*23*, *24*) show that DHFS activity cannot be separated from FPGS activity. The binding studies with the N-terminal domain and $E_{1-287}/L_{299-428}$ and $E_{1-81}/L_{72-82}/E_{93-422}$ mutants show that if a mutant is able to bind DHP, it binds mTHF as well. The point mutations directed at the pteroate binding site affected both the FPGS and the DHFS activity in a very similar manner. The best examples are EcT122H and EcA155H, which greatly increased the K_m values for both DHP and THF substrates.

C-Terminal Domain Is Required for ATP Binding and Glutamate Binding. The binding studies with the *E. coli* N-terminal domain and the domain switch chimera mutants suggest that the C-terminal domain is not required for pteroate binding and does not determine the pteroate/folate specificity of the enzyme. The finding that the domain exchange mutant $E_{1-287}/L_{299-428}$ was inactive and was not able to bind ATP in the equilibrium dialysis experiment suggests that the C-terminus is required and that the interface between the two domains is the binding surface for the substrates that allows catalysis to occur.

It has been reported by Bertrand et al. (*25*, *26*) that *L. casei* FPGS shares significant structural homology with UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase (MurD), an enzyme that catalyzes the addition of D-glutamate to the nucleotide precursor, uridine-diphospho-*N*-acetylmuramic acid, in the murein biosynthetic pathway (*25*). Both proteins have the same topology in their ATP binding and glutamate binding motifs (*25*). In the *L. casei* crystal structure, the glutamate binding site is not refined. A structural comparison with the MurD-UDP-*N*-acetylmuramoyl-L-alanine-D-glutamate (the final product of MurD) complex provides evidence that the C-terminal domain binds glutamate. The site-directed mutagenesis data also support this notion. Mutants S412A (*22*) in the C-terminal of *L. casei* FPGS and H338A in the C-terminal domain human FPGS (*27*) and A309I (*23*) in *E. coli* FPGS resulted in large increases in the K_m value for glutamate. In this study, we show that Y414A in the C-terminal domain of the *L. casei* FPGS also has a large increase in the glutamate K_m value. All these residues are conserved in all FPGS enzymes and are proximal to one another in a cleft between the N- and the C-terminal domains, which has been predicted to be the glutamate binding site by X-ray crystallography (*13*) and computational (*14*) studies.

DHP Binding Site Is Also the Primary mTHF Binding Site in L. casei FPGS. The binding pocket corresponding to the *E. coli* FPGS pteroate binding site also exists in *L. casei* FPGS and was independently predicted by Tan and Carlson (*14*). Their modeling gave more favorable docking energies for all folate substrates to the pteroate binding site, as compared to the mTHF binding site. The D151A and T119W mutants, directed to the pteroate binding site in *L. casei* FPGS, both had a greater than 100-fold decrease in enzyme activity with the effect mainly on the V_{max} value in the case of D151 and the K_m value for folate in the case of T119,

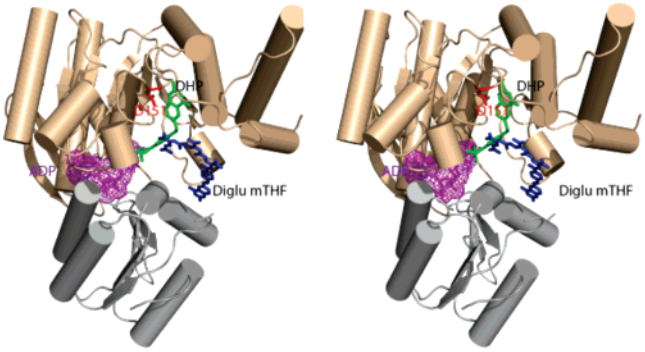

FIGURE 5: Stereoview diagram of the *L. casei* FPGS showing the DHP and the predicted mTHF diglutamate binding sites. The D151 residue (red) is shown in proximity to the pterin of DHP (green), and mutation has a strong effect on its activity. mTHF diglutamate (dark blue) can bind productively to the mTHF binding site as shown. The D151 residue is more distal to the mTHF diglutamate, and its mutation has a 40-fold lesser effect than with THF monoglutamate.

strongly suggesting that the pteroate binding site is the site of mTHF binding in *L. casei* FPGS.

mTHF Binding Site in L. casei FPGS May Be Used To Bind Polyglutamate Substrates. mTHF monoglutamate oriented as in the ternary crystal structure would not be able to effect catalysis because its terminal glutamate moiety is too distant from the bound ATP. The results support the suggestion by Tan and Carlson (*14*) that the mTHF binding site might be utilized by mTHF polyglutantate substrates. We found that the D151A mutant had a much higher activity with mTHF diglutamate as a substrate than with monoglutamate, consistent with the diglutamate binding to the mTHF binding site, which is not affected by the mutation. The D154A mutation of *E. coli* FPGS affects the apparent K_m value for THF monoglutamate and THF diglutamate, to the same extent, suggesting that they bound to the same site, namely, the pteroate binding site. If THF and DHP bind to the same site, this suggests that selective inhibition of DHFS activity may be difficult to achieve and would not be a promising strategy for the rational design of antibiotics to combat bacterial infections.

Pteroate Binding Loop Is Not Essential for Dihydropteroate Binding. In contrast to the suggestion of Mathieu et al. (*15*) that residues 24−32 of the *E. coli* enzyme, particularly the IDLGL sequence from residues 28−32, might be unique to DHFS enzymes, we note that deletion of the first 28 residues in *E. coli* FPGS-DHFS does not inactivate either the FPGS or the DHFS activity (*23*) and that deletion of the corresponding region of *L. casei* FPGS also did not abrogate its activity. We do not, therefore, believe that this loop is essential for DHFS substrate binding. We tested as to whether the loop could confer DHFS activity to *L. casei* FPGS. However, when we constructed *L. casei* FPGS mutated to have the *E. coli* FPGS pteroate binding loop, the mutant enzyme did not use DHP as a substrate, nor did it lose its FPGS activity. We were unable to determine as to which specific residues confer DHFS activity to the *E. coli* FPGS-DHFS enzyme.

Pteroate Specificity Is Not Determined by the Omega Loop or the Pteroate Binding Loop. Both the C-terminal domain deletion mutants and the chimera proteins provided evidence that the N-terminal domain is sufficient for binding folate and for determination of the folate substrate specificity. The omega loop exchange studies showed that $L_{1-71}/E_{82-92}/L_{83-428}$, which contains the *E. coli* FPGS omega loop, still has the characteristics of *L. casei* FPGS, while $E_{1-81}/L_{72-82}/E_{93-422}$, which has the *L. casei* FPGS omega loop, retains bifunctional enzyme activity. However, fluorescence studies showed that $L_{1-71}/E_{82-92}/L_{83-428}$ was able to bind DHP weakly with a K_d value that is 30-fold higher than that of the WT protein. This was the only mutation we studied that changed the pteroate versus folate specificity of the enzyme. These data suggest that the omega loop does contribute to folate substrate specificity determination but that intact 3-D structure elements surrounding the omega loop are more important.

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SUPPORTING INFORMATION AVAILABLE

Table of oligonucleotides used to construct the mutants described in the paper and two figures of polyacrylamide gels showing the purification of two mutants and their yield and purity as compared to the WT. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. McGuire, J. J., and Coward, J. K. (1984) Pteroylpolyglutamates: Biosynthesis, degradation, and function, in *Folates and Pterins* (Blakley, R. L., and Benkovic, S. J., Eds.) pp 135−190, Wiley, New York.
2. Shane, B. (1989) Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism, *Vitam. Horm. 45*, 263−335.
3. Snell, E. E., and Peterson, W. H. (1940) Growth factors for bacteria. X. Additional factors required in certain lactic acid bacteria, *J. Bacteriol. 39*, 2696−2698.
4. Pyne, C., and Bognar, A. L. (1992) Replacement of the *folC* gene encoding folylpolyglutamate synthetase-dihydrofolate synthetase in *Escherichia coli* with genes mutagenized in vitro, *J. Bacteriol. 174*, 1750−1759.
5. Cherest, H., Thomas, D., and Surdin-Kerjan, Y. (2000) Polyglutamylation of folate coenzymes is necessary for methionine biosynthesis and maintenance of intact mitochondrial genome in *Saccharomyces cerevisiae*, *J. Biol. Chem. 275*, 14056−14063.
6. Bayly, A. M., Berglez, J. M., Patel, O., Castelli, L. A., Hankins, E. G., Coloe, P., Hopkins Sibley, C., and Macreadie, I. G. (2001) Folic acid utilization related to sulfa drug resistance in *Saccharomyces cerevisiae*, *FEMS Microbiol. Lett. 204*, 387−390.
7. Shane, B., Bognar, A. L., Goldfarb, R. D., and LeBowitz, J. H. (1983) Regulation of folylpoly-γ-glutamate synthesis in bacteria: In vivo and in vitro synthesis of pteroylpoly-γ-glutamates by *Lactobacillus casei* and *Streptococcus faecalis*, *J. Bacteriol 153*, 316−325.
8. Bognar, A. L., and Shane, B. (1983) Purification and properties of *Lactobacillus casei* folylpolyglutamate synthetase, *J. Biol. Chem. 258*, 12574−12581.
9. Bognar, A., and Shane, B. (1986) Bacterial folylpoly-γ-glutamate synthetase-dihydrofolate synthetase, *Methods Enzymol. 122*, 349−359.
10. Bognar, A. L., Osborne, C., Shane, B., Singer, S. C., and Ferone, R. (1985) Folylpoly-γ-glutamate synthetase-dihydrofolate synthetase cloning and high expression of the *Escherichia coli folC* gene and purification and properties of the gene product, *J. Biol. Chem. 260*, 5625−5630.
11. Masurekar, M., and Brown, G. M. (1975) Partial purification and properties of an enzyme from *Escherichia coli* that catalyzes the conversion of glutamic acid and 10-formyltetrahydropteroylglutamic acid to 10-formyltetrahydropteroyl-γ-glutamylglutamic acid, *Biochemistry 14*, 2424−2430.

12. Sun, X., Bognar, A. L., Baker, E. N., and Smith, C. A. (1998) Structural homologies with ATP- and folate-binding enzymes in the crystal structure of FPGS, *Proc. Natl. Acad. Sci. U.S.A. 95*, 6647−6652.
13. Sun, X., Cross, J. A., Bognar, A. L., Baker, E. N., and Smith, C. A. (2001) Folate-binding triggers the activation of folylpolyglutamate synthetase, *J. Mol. Biol. 310*, 1067−1078.
14. Tan, X.-J., and Carlson, H. A. (2005) Docking studies and ligand recognition in folylpolyglutamate synthetase, *J. Med. Chem. 48*, 7764−7772.
15. Mathieu, M., Debousker, G., Vincent, S., Viviani, F., Bamas-Jacques, N., and Mikol, V. (2005) *Escherichia coli FolC* structure reveals an unexpected dihydrofolate binding site providing an attractive target for anti-microbial therapy, *J. Biol. Chem. 280*, 18916−18922.
16. Blakley, R. L. (1960) Crystallization of dihydropteroylglutamic acid, *Nature (London, U.K.) 188*, 231−232.
17. Kisliuk, R. L., Gaumont, Y., Lafer, E., Baugh, C. M., and Montgomery, J. A. (1981) Polyglutamyl derivatives of tetrahydrofolate as substrates for *Lactobacillus casei* thymidylate synthase, *Biochemistry 20*, 929−934.
18. Shane, B. (1980) Pteroylpoly(γ-glutamate) synthesis by Corynebacterium species, purification and properties of folylpoly(γ-glutamate) synthetase, *J. Biol. Chem. 255*, 5655−5662.
19. Nixon, A. E., Ostermeier, M., and Benkovic, S. J. (1998) Hybrid enzymes: Manipulating enzyme design, *Trends Biotechnol. 16*, 258−264.
20. Sheng, Y., Cross, J. A., Shen, Y., Smith, C. A., and Bognar, A. L. (2002) Mutation of an essential glutamate residue in folylpolyglutamate synthetase and activation of the enzyme by pteroate binding, *Arch. Biochem. Biophys. 402*, 94−103.
21. Smith, C. A., Cross, J. A., Bognar, A. L., and Sun, X. (2006) Mutation of Gly51 to serine in the P-loop of *Lactobacillus casei* folylpolyglutamate synthetase abolishes activity by altering the conformation of two adjacent loops, *Acta Crystallogr. 62*, 548−558.
22. Sheng, Y., Sun, X., Cross, J. A., Shen, Y., Bognar, A. L., Baker, E. N., and Smith, C. A. (2000) Structural and functional similarities in the ADP-forming amide bond ligase superfamily: Implications for a substrate-induced conformational change in folylpolyglutamate synthetase, *J. Mol. Biol. 302*, 427−440.
23. Keshavjee, K., Pyne, C., and Bognar, A. L. (1991) Characterization of a mutation affecting the function of *Escherichia coli* folylpolyglutamate synthetase-dihydrofolate synthetase and further mutations produced in vitro at the same locus, *J. Biol. Chem. 266*, 19925−19929.
24. Kimlova, L. J., Pyne, C., Keshavjee, K., Huy, J., Beebakhee, G., and Bognar, A. L. (1991) Mutagenesis of the *folC* gene encoding folylpolyglutamate synthetase-dihydrofolate synthetase in *Escherichia coli*, *Arch. Biochem. Biophys. 284*, 9−16.
25. Bertrand, J. A., Auger, G., Martin, L., Fanchon, E., Blanot, D., Le Beller, D., van Heijenoort, J., and Dideberg, O. (1999) Determination of the MurD mechanism through crystallographic analysis of enzyme complexes, *J. Mol. Biol. 289*, 579−590.
26. Bertrand, J. A., Fanchon, E., Martin, L., Chantalat, L., Auger, G., Blanot, D., van Heijenoort, J., and Dideberg, O. (2000) "Open" structures of MurD: Domain movements and structural similarities with folylpolyglutamate synthetase, *J. Mol. Biol. 301*, 1257−1266.
27. Sanghani, S. P., Sanghani, P. C., and Moran, R. G. (1999) Identification of three key active site residues in the C-terminal domain of human recombinant folylpoly-γ-glutamate synthetase by site-directed mutagenesis, *J. Biol. Chem. 274*, 27018−27027.

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