Binding of ATP as Well as Tetrahydrofolate Induces Conformational Changes in *Lactobacillus casei* Folylpolyglutamate Synthetase in Solution†

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ABSTRACT: Folylpolyglutamate synthetase (FPGS) catalyzes the addition of glutamate to folate derivatives to form folate polyglutamates. FPGS is essential for folate biosynthesis in bacteria and retention of folate pools in eukaryotes. X-ray crystallographic analyses of binary and ternary complexes of *Lactobacillus casei* FPGS suggest that binding of folate triggers a conformational change that activates FPGS. We used EPR and CD spectroscopy to further characterize the conformational change in the FPGS reaction. For EPR spectroscopy, two cysteine residues were introduced into FPGS by site-directed mutagenesis, K172C in the N-terminal domain and D345C in the C-terminal domain. The mutant protein was expressed, purified, and labeled with methanethiosulfonate. Addition of ATP, tetrahydrofolate, or 5,10-methylenetetrahydrofolate but not glutamate to FPGS showed broadening of EPR spectra, which is due to stronger spin−spin interactions, suggesting that both ATP and tetrahydrofolates cause a conformational change. ATP binding had an EPR spectrum distinct from that of tetrahydrofolate binding, indicating that it caused a different conformational change. When both ATP and THF were bound, the spectrum was identical to that seen when THF alone bound to the enzyme, showing that the THF-induced conformation was dominant. The spectral broadening suggests that the conformation change involves the two domains moving closer together, which is consistent with the rigid-body rotation of the C-terminal domain observed in the FPGS crystal structure with AMPPCP and 5,10-methylenetetrahydrofolate bound. No changes in the CD spectra were observed with the addition of FPGS substrates, suggesting that the conformational changes did not affect the secondary structure elements of the enzyme. These studies confirm the conformational change seen in the crystal structure by an independent method but also show that ATP binds to the free enzyme and affects its conformation.

Folate coenzymes act as acceptors and donors of one-carbon units in reactions involved in amino acid and nucleotide metabolism in the cell. Folates exist in cells primarily as poly-γ-glutamate derivatives. Folate polyglutamates often have a higher affinity for folate-dependent enzymes and therefore are more effective as substrates or inhibitors (*1*, *2*). For organisms that require exogenous folate for growth, such as *Lactobacillus casei* and mammals, polyglutamylation serves as a mechanism to accumulate and maintain cellular folate pools. The polyglutamylation reaction is catalyzed by the enzyme folylpolyglutamate synthetase (FPGS),[1] which is present in all cells. The importance of FPGS in folate metabolism has made it a promising candidate for targeting in antimicrobial and cancer chemotherapy (*3*−*5*). Substrate activity with FPGS is required for the retention and clinical efficacy of antifolate drugs, which require transport to enter eukaryotic cells (*3*−*6*). As a result, understanding the determinants of folate binding and the catalytic mechanism of FPGS is important for the design of novel antifolate drugs.

FPGS catalyzes the ATP-dependent addition of a glutamate residue via an amide linkage to the γ-carboxylate group of the folate or folate polyglutamates. The reaction mechanism involves the activation of the free carboxylate group of folate by ATP, forming an acyl phosphate intermediate, followed by nucleophilic attack by the amino group of the incoming glutamate to form the glutamylated folate. X-ray crystallographic data of the binary and ternary complexes of *L. casei* FPGS with its substrates (*7*, *8*) suggested that a conformational change occurred in response to folate binding. Our recent biochemical study of FPGS (*9*) used equilibrium dialysis and fluorescence studies to show a dramatic increase in ATP binding to *Escherichia coli* FPGS upon dihydropteroate or tetrahydrofolate binding. This was also consistent with a conformational change triggered by folate binding. Although a conformational change has been suggested by both these studies, some important questions still need to be addressed to fully understand the mechanism and catalysis of FPGS. For example, it is not known whether the

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[1] Abbreviations: AMPPCP, β,γ-methylene-ATP; CD, circular dichroism; DDATHF, (6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolate; EPR, electronic paramagnetic resonance; FPGS, folylpolyglutamate synthetase; MTSL, 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate; SDSL, site-directed spin labeling; THF, (6*RS*)-5,6,7,8-tetrahydrofolate, T_m, the midpoint temperature of denaturation in differential scanning calorimetry.

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conformational change requires the presence of ATP or whether FPGS undergoes another conformational change upon binding the third substrate, glutamate. We address these questions in the present work.

Biophysical methods, such as CD and EPR, have been widely used to detect conformational changes in various proteins, because they are easy to perform and often do not interfere with the reaction. CD is a sensitive method for detecting conformational changes that involve protein secondary structure elements. EPR has the advantage of being able to examine global protein structure and dynamics. In particular, site-directed spin labeling (SDSL), in which site-directed mutagenesis is used to introduce spin-labeled cysteine at desired sites in a protein, has been developed as a tool to study protein structure, side chain accessibility and mobility, and changes in the distance between two labeled sites (*10*). It greatly expands the application of EPR. It overcomes the limitations of both the X-ray crystallographic analyses and NMR. The former does not provide a dynamic picture, and the latter only applies to low-molecular-weight soluble proteins. In this work, we systematically characterize substrate-induced conformational changes of FPGS using both CD and SDSL methods.

MATERIAL AND METHODS

Materials. (6*RS*)-5,6,7,8-Tetrahydrofolate and (6*RS*)-5,10-methylenetetrahydrofolate were obtained from Schircks Laboratories, Switzerland. The spin label 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate (MTSL) was from Toronto Research Chemicals, Canada.

Site-Directed Mutagenesis and Protein Expression and Purification. Site-directed mutagenesis was performed directly on the recombinant expression plasmid pCYB1 (NEB) encoding *L. casei* FPGS as a fusion protein with an intein and a chitin binding domain. Oligonucleotide primers used for site-directed mutagenesis are as follows: K172C, 5′-GCT TTA GAT CAC CAG TGT TTG CTG GGG CAT ACG-3′, 5′-CGT ATG CCC CAG CAA ACA CTG GTG ATC TAA AGC-3′; D345C, 5′-GCC GGC ATC TTG GCG TGT AAA GAC TAT GCG GCG-3′, 5′-CGC CGC ATA GTC TTT ACA CGC CAA GAT GCC GGC-3′. The single mutations K172C and D345C were introduced by the oligonuclotide primer pairs using the Quick Change site-directed mutagenesis procedure of Stratagene. The double-mutant K172C/D345C was constructed by digestion of the single mutants with *Hin*dIII and replacement of the wild-type sequence in the *Hin*dIII fragment of the K172C mutant with the corresponding fragment of the D345C mutant. All mutations were confirmed with DNA sequencing.

The FPGS wild-type and mutant proteins were expressed and purified with the IMPACT one-step purification system (NEB) as described previously (*9*). The purified proteins were dialyzed against 10 mM Tris−HCl, pH 7.5, 200 mM KCl, and 10 mM $MgCl_2$ and stored at −70 °C with 20% DMSO. Protein concentration was determined using a UV spectrophotometer (Beckman DU640) by the absorbance at 280 nm using an absorbance coefficient of 52170 (mol^{-1} cm^{-1}) for FPGS (*11*).

FPGS Enzyme Activity Assay. Enzyme activities were measured by the incorporation of [^{3}H]glutamate into 5,10-methylenetetrahydrofolate or tetrahydrofolate as described by Shane (*12*). A standard *L. casei* FPGS assay mixture consisted of 100 mM Tris, 50 mM glycine, pH 9.75, 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, 200 μM tetrahydrofolate, 12 mM formaldehyde, and 5−10 μg of pure enzyme. For the assay with spin-labeled enzyme and the mock-labeled wild-type control, DTT was omitted from the reaction mixture.

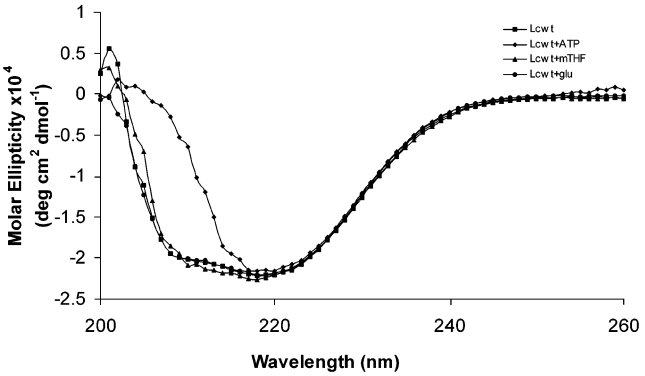

FIGURE 1: Far-UV CD spectra of free and substrate-bound *L. casei* FPGS. The CD spectra of protein alone (■) and with ATP (◆), 5,10-methylene-THF (▲), and glutamate (●) were determined as described in the Materials and Methods.

CD Spectroscopic Measurement. CD measurements were conducted on an AVIV 62DS circular dichroism spectrometer. Far-UV CD spectra were recorded in a 0.1 cm quartz cell in a buffer containing 10 mM Tris−HCl, pH 7.5, 250 mM KCl, and 10 mM $MgCl_2$ at room temperature. Measurements were read from 260 to 200 nm in 1 nm intervals with a 1 s averaging time. The spectrum of a buffer blank with or without the corresponding substrate was subtracted from the protein spectrum with or without substrate. Each experiment is the average of 10 scans.

Spin Labeling. The FPGS single- and double-mutant proteins were labeled with MTSL spin label. The FPGS mutant proteins (15 μM) were first incubated with 20 mM DTT for 1 h at room temperature. DTT was removed via extensive dialysis against buffer containing 10 mM Tris−HCl, pH 8.0, 250 mM KCl, 10 mM $MgCl_2$, and 10% DMSO at 4 °C. Spin labeling was accomplished by incubating 15 μM protein with 300 μM MTSL overnight at 4 °C. The free label was removed by extensive dialysis against the same buffer as described above. The protein was concentrated using a centrifugal filter unit (5K, Millipore) to between 200 and 400 μM.

EPR Measurement and Analysis of Spectra. EPR spectra were obtained on an Elexsys E500 spectrometer (Bruker, Rheinstetten, Germany). The conventional X-band EPR spectra were recorded using 25 dB microwave power and a modulation amplitude of 1.6 G at room temperature. Spin population was standardized by double integration using the software package Xport provided by Bruker Inc.

RESULTS

CD Spectroscopy of FPGS. We first tested whether substrate binding caused a change in the secondary structure of FPGS by CD analysis (Figure 1). The far-UV CD spectra of FPGS with 1 mM glutamate or 200 μM 5,10-methylene-THF were very similar to the spectrum of free FPGS,

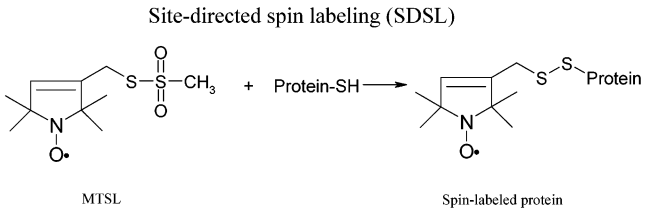

FIGURE 2: Reaction of MTSL spin label with the sulfhydryl of a cysteine residue on a protein to generate a nitroxide-containing side chain.

indicating that the enzyme had similar secondary structure or backbone conformation in the presence or absence of these substrates. Due to high absorbance in the samples containing ATP, the spectrum of FPGS with ATP below 219 nm cannot be accurately measured. At 220 nm, the ellipticity of the spectrum of FPGS with ATP had the same value as that of the spectrum of free FPGS, suggesting that ATP did not affect the secondary structure of FPGS (Figure 1).

Construction and Function of Mutant FPGS. To examine the conformational changes FPGS undergoes upon binding its substrates, two residues, Lys172 and Asp345, were mutated to cysteine and labeled with MTSL, a nitroxide spin label with strict specificity for free thiol groups (Figure 2). These two residues were chosen for the following reasons: First, Lys172 and Asp345 are located on the N-terminal and C-terminal domains of FPGS, respectively. Therefore, it is possible to detect domain movement through the spin−spin interactions of these two sites. Second, they are both hydrophilic residues on the surface of the protein and are presumably more accessible to the spin label reagent. Third, they are not directly located at the active site of FPGS according to the X-ray structure of FPGS and are not likely to interfere with substrate binding. The effect of a mobility change in a single residue upon substrate binding can be easily distinguished from the effect of a change in distance between these two sites. Last, these two residues are close enough to each other for an interaction to be detected using EPR, which is sensitive at distances below 30 Å. The wild-type *L. casei* FPGS has no cysteine residues that could react with the spin label to complicate our study.

Figure 3 shows the structure of FPGS bound to the ATP analogue AMPPCP and 5,10-methylene-THF (the ternary structure) with the Lys172 and Asp345 highlighted superimposed with the structure of the apoprotein. In the ternary structure, the distance between the carboxyl carbon of Asp345 and the ϵ-amino group of Lys172 is 10.95 Å, and that between the C-α atoms of these two residues is 10.77 Å. Both residues are located in a loop region. In the apoprotein the structure around both Lys172 and Asp345 is not defined, and therefore the exact distance between these two residues cannot be provided. However, A350, the nearest residue that does appear in the binary structure, is 6.13 Å farther from Leu168 in the apoprotein than in the ternary complex and 3.44 Å farther from Leu168 than in the structure of FPGS complexed with ATP alone (the binary structure; *8*), suggesting that ATP binding also has a conformational effect on the residues in this region.

Enzyme activity was examined for both single and double mutants of FPGS to ensure that the mutant proteins are fully active and can represent the function of the wild-type protein. Table 1 shows the relative enzyme activities of the wild-type and mutant FPGS proteins. Using 5,10-methylene-THF, the best folate substrate for FPGS, all mutant enzymes have more than 85% wild-type enzyme activity, suggesting mutations have little effect on enzyme function. We used both 5,10-methylene-THF and THF as the folate substrates in EPR measurement. THF is not as effective a substrate for *L. casei* FPGS as 5,10-methylene-THF. The K_m for THF of the wild-type FPGS is 1 mM, compared with 30 μM for 5,10-methylene-THF. THF and 5,10-methylene-THF are very labile in solution, and 5,10-methylene-THF is also unstable and breaks down to formaldehyde and THF. The amount of 5,10-methylene-THF remaining after the experiment was spectrally determined and found to be 50% of the amount weighed out. We normally use β-mercaptoethanol or ascorbate for stabilization and storage of reduced folates. However, both these antioxidants either cause cleavage of the spin label from the protein or diminish the EPR signal. Therefore, fresh THF was prepared every time to avoid degradation of THF. In the enzyme assay, fresh THF, assayed without DTT, was used to test mock-labeled wild-type enzyme (the wild-type FPGS which had gone through the same labeling procedure as the mutant proteins) and the labeled mutants. All the mutants showed between 80% and 100% wild-type activity, indicating the mutant proteins were fully functional and labeling did not affect them.

EPR Spectroscopy of FPGS. The spin−spin interaction of the K172C/D345C double-mutant protein is readily detected by an amplitude decrease and line width increase in its EPR spectrum when the spectrum of the double mutant is compared to the sum of the spectra of the single-mutant proteins (K172C and D345C) (Figure 4). The mechanism of the spin−spin interaction under the experimental conditions used here was mainly weak exchange through space (*13*), static−dipolar interactions (*14*), and modulation of the dipolar interaction by molecular tumbling. Each of these effects leads to distance-dependent broadening of the EPR spectrum in an isotropic distribution of nitroxides (*15*).

Figure 5 shows the EPR spectra for the spin-labeled double-mutant protein with and without substrate. Broadened EPR spectra can be seen in the ATP (6 mM) and THF (1 mM) bound double-mutant protein but not the glutamate (1 mM) bound form. The spectra of the ATP-bound and the THF-bound proteins are distinct as shown in Figure 5d.

The broadening effect can come from two sources: spin−spin interaction and a decrease in the mobility of the nitroxide side chain. To examine whether substrate binding can cause a mobility change of the spin label side chain, we measured the EPR spectra of each single-mutant protein with and without substrate. As shown in Figure 6, the spectra of K172C and D345C with their substrate ATP or THF can be completely superimposed with the spectra of the unbound single-mutant protein, suggesting neither ATP nor THF can cause a mobility change of the side chains of either single-mutant protein. Therefore, the spectral broadening that occurred upon the addition of ATP or THF to the spin-labeled FPGS double-mutant protein must be the result of an increase in spin−spin interactions, which indicates a movement of the two sites closer together.

To further characterize the broadening effect by ATP and THF, the amount of ATP and THF was varied in EPR measurement. The K_m for ATP of *L. casei* FPGS is 3 mM, for 5,10-methylene-THF it is 30 μM, and for THF it is 1

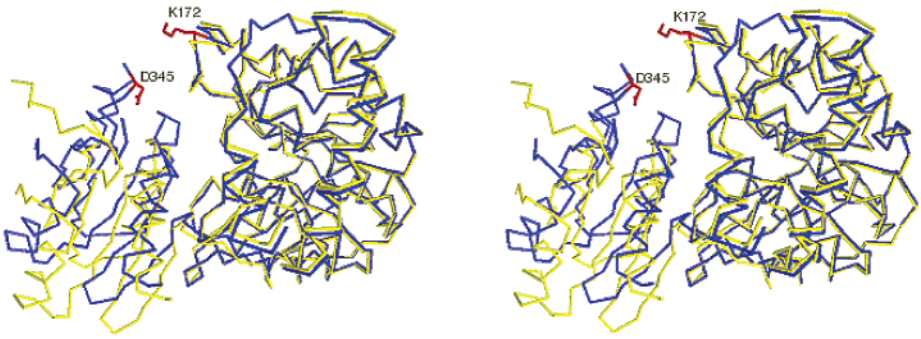

FIGURE 3: Superposition of the ternary FPGS−AMPPCP−mTHF complex (blue) with the apoprotein (yellow). The two mutated residues (red) K172 and D345 are highlighted.

Table 1: Relative Activity of *L. casei* FPGS Wild-Type and Mutant Proteins[a]

unlabeled			spin-labeled		
sample	enzyme activity[b] [(nmol/mg)/h]	relative activity (%)	sample	enzyme activity[c] [(nmol/mg)/h]	relative activity (%)
wild type	12000	100	wild type	3643	100
K172C	11000	98	K172CMTSL	3400	93
D345C	10000	83	D345CMTSL	3280	90
K172C/D345	10560	88	K172C/D345	3020	83

[a] FPGS activity was assayed as described in the Materials and Methods. [b] The activity of *L. casei* FPGS and mutant proteins without labeling was determined using 5,10-methylenetetrahydrofolate as the substrate. [c] The activity of *L. casei* FPGS and mutant proteins with labeling was determined using tetrahydrofolate as the substrate in the absence of DTT or mercaptoethanol. The wild-type protein assayed here went through the same labeling procedure as the mutant proteins.

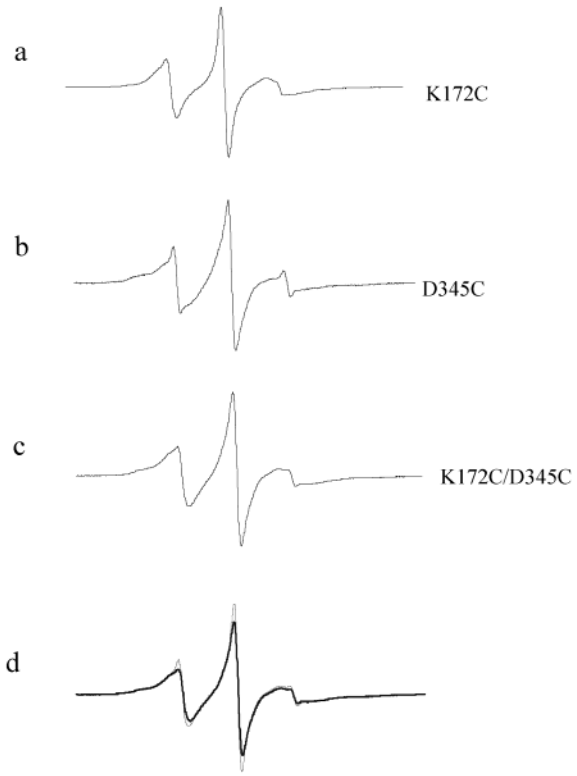

FIGURE 4: EPR spectra of MTSL-labeled single-mutant proteins K172C (a) and D345C (b) and the double-mutant K172C/D345C (c). The spectrum of the double mutant (heavy trace) superimposed with the sum of the single mutants (light trace) is shown in (d). The spectra were recorded at room temperature with a total scan of 100 G. All spectra were normalized to represent of the same number of spins.

mM. We found that 3 mM ATP did not cause a significant change in the EPR spectrum (data not shown), while 6 mM ATP broadened the spectrum of the double mutant compared with the spectrum of the sum of the single mutants with the same amount of substrates (not shown). A significant change was caused by 1 mM THF, which was almost a 2-fold decrease in amplitude and increase in line width. The effect of broadening in EPR spectra with THF was dose-dependent as shown in Figure 7a. 5,10-Methylene-THF produced a significant line broadening at 50 μM, which is just higher than its K_m, and the broadening was also dose-dependent (Figure 7b). The spectrum of enzyme bound to 5,10-methylene-THF was qualitatively similar to that of enzyme bound to THF, showing that these substrates induce the same conformational change. The amplitude of the broadening effect with 5,10-methylene-THF in the figure is not directly comparable to the effect with THF because the protein concentration in the experiment with 5,10-methylene-THF was about half that of the experiment with THF. With 5,10-methylene-THF we did not see saturation because of the instability of the substrate. However, the broadening effect was seen at a much lower concentration of 5,10-methylene-THF than THF, reflecting the difference in K_m of the substrates. We were unable to use saturating concentrations of THF or ATP because of their high K_m values. However, the broadening effect by 0.5 mM THF (half its K_m) was about the same magnitude as that caused by 6 mM ATP (twice the K_m). The spectrum obtained when both ATP (6 mM) and THF (1 mM) were added to the enzyme was virtually identical to the spectrum with THF (1 mM) alone (Figure 7c) but was not identical to the spectrum with ATP alone. The above data suggest that a qualitatively different conformational change occurred with ATP, compared to that caused by THF or 5,10-methylene-THF. The conformation that resulted after THF binding appeared to be dominant over that caused by ATP binding. THF did not require ATP binding for its effect, indicating that it can bind to free enzyme and cause it to change its conformation.

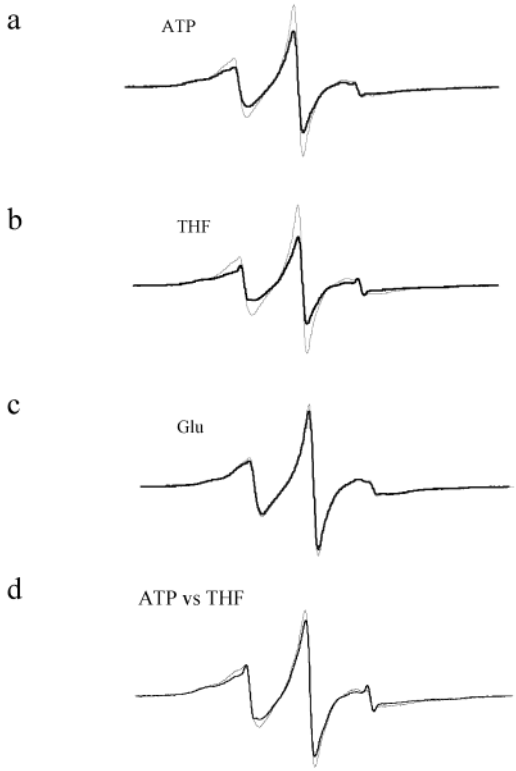

FIGURE 5: EPR spectra of the MTSL-labeled K172C/D345C double-mutant protein in the absence and presence of ligands. EPR spectra show the amplitude of signal in arbitrary units versus field strength. The spectra of the spin-labeled double-mutant protein with (a) no ligand (light trace) and with ATP (heavy trace), (b) no ligand (light trace) and with THF (heavy trace), and (c) no ligand (light trace) and with glutamate (heavy trace) are shown superimposed. In (d), the spectrum of the double-mutant protein with ATP bound (light trace) is shown superimposed on the same protein with THF bound (heavy trace).

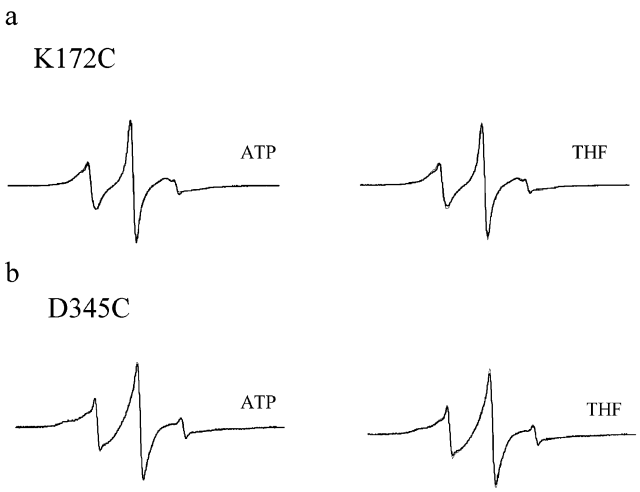

FIGURE 6: EPR spectra of the MTSL-labeled single-mutant proteins K172 C (a) and D345C (b) in the absence and presence of substrates. The spectra of the single-mutant protein without substrate (light trace) and with ATP (heavy trace) are shown superimposed on the left. The spectra of the single mutant without substrate (light trace) and with THF (heavy trace) are shown superimposed on the right.

DISCUSSION

The high-resolution crystallographic analyses of the binary complexes of *L. casei* FPGS with ATP (*7*) or AMPPCP and the ternary complex with ACP4- and 5,10-methylene-THF

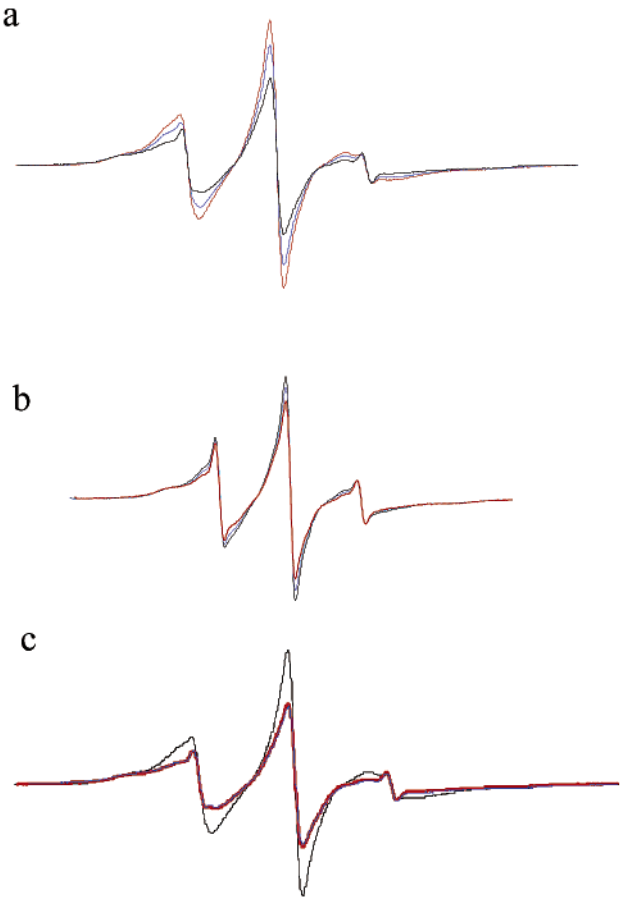

FIGURE 7: (a) EPR spectra of the MTSL-labeled K172C/D345C double-mutant protein in the absence of ligand (red) or with 0.5 mM (blue) and 1 mM (black) THF. (b) EPR spectra of the MTSL-labeled K172C/D345C double-mutant protein in the absence of ligand (red) or with 50 μM (blue) and 200 μM (black) 5,10-methylene-THF. (c) EPR spectrum of the MTSL-labeled K172C/D345C double-mutant protein in the absence or presence of ligand. The spectrum of the double mutant without ligand is shown in black, that in the presence of 1.0 mM THF in red, and that in the presence of both 1.0 mM THF and 6 mM ATP in blue.

(*8*) suggested that the binding of ATP to free FPGS was not productive or sufficient to generate an active enzyme. In the X-ray crystal structure of the FPGS−MgATP (*7*) complex the positions of the adenosine and the γ-phosphate of ATP could not be resolved, suggesting that the cofactor was nonspecifically bound. Binding of folate as the second substrate triggered a large-scale rigid-body rotation (about 20°) of the C-terminal domain. The ATP became more specifically bound by insertion of its adenosine moiety into the deep cleft between the N- and C-terminal domains and by adoption of a structurally favorable anti configuration following rotation of the C-terminal domain (*8*). X-ray crystallographic analyses provide a detailed static picture of the conformational change that occurs in FPGS upon binding ATP and folate. This picture may not represent the conformation of the enzyme in solution. Our present work was aimed at confirming this conformational change of FPGS by an independent method.

Our CD results indicated that the conformational changes did not involve a secondary structure change in FPGS, consistent with the X-ray structural analyses. The EPR study confirmed that the conformational change of FPGS was

caused by the movement of the N- and C-terminal domains of FPGS closer to each other.

The EPR results also showed that not only THF but also ATP could trigger a conformational change. The X-ray structural data showed little difference between the overall structure of the FPGS apoprotein and the protein complexed with ATP (*7*) or AMPPCP (*8*). However, when we measured the distance between the nearest residues and those that we spin-labeled with structural assignments in the apoprotein, there was a significant difference between the apoprotein and the binary FPGS−AMPPCP complex. Thus, we may have chosen a position to label, which does change its conformation with ATP binding.

From our EPR study, we found that ATP can trigger a conformational change while THF or 5,10-methylene-THF activates a distinct conformational change. The fact that the EPR spectrum of the enzyme binding both ATP and THF is similar to that of the enzyme binding THF alone suggests that the THF is the dominant force for the conformational change of FPGS. This is consistent with ATP binding to the free enzyme being nonproductive and requiring another conformational change upon folate binding to activate the enzyme as suggested by the X-ray crystallographic data.

The conformational change caused by THF is greater at the same level of saturation than that caused by ATP. This means that ATP causes a smaller conformational change, supporting the predictions of X-ray crystallography. The X-ray crystallographic data predict that THF causes a conformational change in FPGS to a more closed form. The EPR data support this prediction, since the residues chosen would come closer together if the gap between the N- and C-terminal domains became more closed. This suggests that at least two conformers exist in FPGS solution, the open relaxed form and the closed compact form. Without substrate, the relaxed form dominates in equilibrium. Upon substrate binding, THF shifts the equilibrium to the domain-closed form.

Our study also shows that the conformational change does not require the presence of both ATP and THF. Either ATP or THF has an effect on the FPGS conformation in solution, but glutamate does not. This is consistent with glutamate not binding to the free enzyme and requiring the binding of THF to form its binding pocket as predicted by X-ray crystallography.

Differential scanning calorimetry studies (not shown) found no difference in the T_m for denaturation of the enzyme in the presence of glutamate, compared to the free enzyme. ATP binding lowered the T_m by 5 °C from 51 to 46 °C, and 5,10-methylene-THF lowered the T_m to 50.6 °C. Binding of both 5,10-methylene-THF and ATP resulted in a T_m of 44.7 °C. This supports our conclusion that glutamate does not bind to the free enzyme and that ATP and THF have distinct conformational effects.

These results are at odds with the kinetic mechanism that has been determined for FPGS. The Ter−Ter mechanism proposed for FPGS by the kinetic data suggests that ATP is the first substrate to bind the enzyme, followed by folate and then glutamate (*16*−*20*). In an ordered mechanism one would not expect the second substrate to bind to the free enzyme. However, fluorescence studies with the wild-type *E. coli* FPGS showed that folate binds to the free enzyme with the same affinity as the enzyme−ATP complex (*9*). Here, we show that THF alone can trigger the conformational change that is similar to the effect of ATP and THF together. Together with the binding study, it suggests that the order of binding of the first two substrates may be random. In fact, folate substrate binding seems to be essential for the initiation of catalysis because it causes the conformational change to the domain-closed form. Therefore, THF may effectively be the first substrate to bind. These results suggest we may have to reinterpret the kinetic mechanism for FPGS. The main piece of evidence, which determined the order of the sequential mechanism, was that P_i product inhibition was competitive with ATP and noncompetitive with the other two substrates (*16*). These studies show that ATP could bind the free enzyme and thus could be competitive with P_i. THF may not be able to displace P_i and thus may be noncompetitive with P_i but still be able to bind the free enzyme to cause the conformational change. THF is also competitive with its polyglutamate products as the latter are also substrates for the enzyme and murine FPGS is subject to feedback inhibition by folate polyglutamates. If THF does not require the enzyme−ATP complex to bind to FPGS, it is possible that it may be polyglutamylated processively to long-chain polyglutamates without desorption from its binding pocket. Andreassi and Moran (*21*) suggested that mouse FPGS can add polyglutamates processively to DDATHF but not to aminopterin so the mechanism of the enzyme may vary with the folate substrate used. In this study THF and 5,10-methylene-THF both bound the free enzyme and caused similar conformational changes, so they likely act through the same mechanism.

In vivo *L. casei* FPGS can add up to eleven glutamate moieties to the folate substrates, while in vitro the glutamate chain can reach four with a trace of five. To perform its function, FPGS must have the ability to accommodate different extents of domain movement, depending on the polyglutamate chain length of its folate substrate. The X-ray structural data seem to support this hypothesis. The rotation of the C-terminal domain predicted by the X-ray crystallographic data only involves small changes in the main chain torsion angles of the residues in the interdomain linker, namely, His296, Trp297, and Pro298. The inherent flexibility of the interdomain linker and lack of direct interdomain interactions both favor this rigid-body movement (*8*). Although the transition from the open to the closed form requires a rigid-body rotation of the entire C-terminal domain, this transition may not need a lot of energy. If polyglutamylation requires different extents of domain movement, multiple conformers with similar energies may exist in FPGS solution and binding folate substrate with different glutamate chain lengths may determine which conformer dominates in equilibrium. Further EPR studies using folate substrates with different glutamate chain lengths may be able to address this question.

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