Interaction of Thymidylate Synthase with Pyridoxal 5′-Phosphate As Studied by UV/Visible Difference Spectroscopy and Molecular Modeling†

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ABSTRACT: Pyridoxal 5′-phosphate (PLP) is an effective inhibitor of *Lactobacillus casei* thymidylate synthase (TS), competitive with respect to the nucleotide substrate dUMP (Chen et al., 1989). The UV/vis difference spectra of TS–PLP complexes show λ_{max} at 328 nm due to the specific interaction between Cys 198 of TS and PLP to form a thiohemiacetal, and λ_{min} at 388 nm due to depletion of free PLP. At high concentrations of PLP a new absorbance at 430 nm forms due to nonspecific Schiff base formation between PLP and lysine residues of the enzyme. Using spectral titration at 328 nm, the binding constant of the specific TS–PLP complex was determined to be 0.5 μM, and the stoichiometry was 2 mol of PLP/mol of TS dimer. The 328-nm absorbance of the TS–PLP complex can be competitively and completely eliminated by addition of dUMP or dTMP; this serves as a convenient binding assay for molecules which bind to the active site of TS. Analogs of PLP which do not contain the phosphate or the aldehyde moieties of PLP bound poorly to the enzyme, thus demonstrating the importance of these functional groups for binding. When treated with PLP, C244T TS, which contains the active site Cys 198 as the sole cysteine residue, showed the same properties as the wild-type enzyme. Treatment of the C198A and C198S mutants with PLP did not produce the absorbance at 328 nm assigned to thiohemiacetal formation. Molecular modeling studies showed that when the phosphate of PLP is docked into the phosphate binding site of TS, the aldehyde carbon of PLP is perfectly positioned to form a thiohemiacetal with the catalytic thiol of Cys 198. Side chains of amino acid residues of the protein which are close to or may interact with PLP were tentatively assigned.

Thymidylate synthase (TS;[1] EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2H_4folate to dTMP and H_2-folate. TS has been widely studied, and much is known about the structure, function, and inhibition of the enzyme [cf. Santi and Danenberg (1984)]. The amino acid sequences of TS from 22 organisms are known (Perry et al., 1990; D. Santi, unpublished), and three-dimensional structures of TSs from several sources have been determined (Hardy et al., 1987; Matthews et al., 1990a,b; Montfort et al., 1990; Perry et al., 1990). The catalytic mechanism of TS is well understood and involves nucleophilic attack of the thiol of a conserved cysteine residue (Cys 198 in *Lactobacillus casei*) at C-6 of dUMP to activate the heterocycle for methylation.

Since TS is required for the *de novo* synthesis of dTMP, it has been a popular target for chemotherapeutic agents (Santi & Danenberg, 1984). Most inhibitors of TS are analogs of the substrates dUMP or CH_2H_4folate; however, these analogs have deficiencies as potential therapeutics. In order to gain access across cellular membranes, nucleotides must be provided as the corresponding bases or nucleosides and, therefore, must undergo appropriate metabolic activation. CH_2H_4folate analogs often require conversion to polyglutamylated forms in order to obtain potent inhibition. Selectivity is also an important issue since folate analogs frequently inhibit other enzymes which utilize folate cofactors. From a number of standpoints, it would be desirable to have TS inhibitors which are structurally unrelated to TS substrates.

It has been reported that pyridoxal phosphate (PLP) is an inhibitor of TS, competitive with respect to dUMP (Chen et al., 1989). It was proposed that the inhibition involved binding of the PLP phosphate moiety to the dUMP binding site, and formation of a thiohemiacetal with a thiol group. In the present work, we have expanded on these observations and have performed detailed studies of the UV/vis difference spectra of the complexes formed between *L. casei* TS and several mutants with PLP and several PLP analogs. The results conclusively demonstrate the necessity of enzyme–phosphate interactions and thiohemiacetal formation with Cys 198 for effective inhibition by PLP. Further, molecular modeling studies have been performed to verify the feasibility of these interactions, and to explore other possible interactions between PLP and TS. From such studies, ideas have emerged as to how one might approach the design of new inhibitors of this enzyme.

MATERIALS AND METHODS

Plasmids which express C198A and C198S mutant *L. casei* TSs in the Thy⁻ *Escherichia coli* strain χ2913 have been described (Climie et al., 1990). The C244T mutant was provided by Dr. V. N. S. K. Francis (Francis and Santi, unpublished). All enzymes were purified using automated sequential chromatography on phosphocellulose and hydroxyapatite (Kealey & Santi, 1992) and were >95% homogeneous as judged by Coomassie-stained SDS–PAGE. Pyridoxal-5′-phosphate, pyridoxal hydrochloride, ϵ-aminocaproic acid, dUMP, and dTMP were purchased from Sigma. 5-Deoxypyridoxal was a gift from Dr. D. E. Metzler, University of Iowa, and Dr. M. Cortijo, University of Complutense, Spain. *N*-Methylpyridoxal phosphate was a gift from Dr. J. Kirsch

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[1] Abbreviations: TS, thymidylate synthase; PLP, pyridoxal 5′-phosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, thymidine 5′-monophosphate; H_2folate, 7,8-dihydrofolate; TES, 2-[*N*-[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; βME, β-mercaptoethanol; DTT, dithiothreitol.

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(University of California, Berkeley). The PLP analogs 3-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)propionic acid (Iwata & Metzler, 1967) and pyridoxol phosphate (Morrison & Long, 1958) were synthesized as reported and characterized by NMR and mass spectrometry. All other chemicals and solvents were reagent grade or better.

The standard TES buffer used in titrations and kinetic measurements is composed of 50 mM TES, pH 7.4, 6.5 mM formaldehyde, 1 mM EDTA, and 25 mM $MgCl_2$. Wild-type and mutant TS (~140 μM)were reduced with 2.5 mM DTT for at least 10 min on ice, and excess thiol was removed by passage through a 0.9 × 2.8 cm Sephadex G-25 column (NAP-5, Pharmacia) equilibrated with 50 mM TES, pH 7.4. Steady-state kinetic assays were performed as previously described (Pogolotti et al., 1986). Inhibition analysis assumed that PLP and PLP analogs are competitive inhibitors with respect to dUMP (Chen et al., 1989); K_i values were obtained by varying inhibitor concentration and fitting the resulting initial rates to a modification of eq III-5 described by Segel [Segel (1975), p 105] using the program Kaleidagraph (Abelbeck Software) run on a Macintosh II computer. Enzyme concentrations were determined using $\epsilon_{278} = 1.26 \times 10^5$ M^{-1} cm^{-1} (Santi and Zhao, unpublished data). The concentration of PLP was determined spectrophotometrically; the reported extinction coefficients (ϵ_{295}= 6700 in 0.1 N HCl; ϵ_{388}= 4900 M^{-1} cm^{-1} in 0.1 M sodium phosphate, pH 7.0; and ϵ_{388}= 6600 M^{-1} cm^{-1} in 0.1 N NaOH; Peterson & Sober, 1953) were used to determine an extinction coefficient in the standard TES buffer at pH 7.4 (ϵ_{388} = 5134 M^{-1} cm^{-1} at pH 7.4). Thiol concentrations were determined by reaction with DTNB (Ellman, 1959).

UV/Vis Difference Spectroscopy. Absorbance spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer, and all spectra were corrected for light scattering using the program SCATWAV supplied by Hewlett-Packard. UV/vis difference spectra were obtained using two approaches. Method A provides a spectrum which reflects the difference in absorbance between bound and free PLP and was used to determine K_d values for TS–PLP complexes. Equal increments of a solution of PLP (or analogs) were added to 1.0- or 10.0-cm path length sample cuvettes containing 6 or 0.6 μM enzyme, respectively, and to a reference cuvette containing buffer. Absorbance spectra (250–600 nm) were measured, light scattering corrections were made, and the spectrum of the reference cuvette was subtracted from that of the sample cuvette. Method B yields composite spectra of bound and free PLP and was used when titrating PLP with enzyme, thiols, or amine. In this method, the absorbance spectrum of an equimolar amount of TS, thiol, or amine was subtracted from the spectrum of a mixture of PLP and titrant. Titration data were fit to a modification of an equilibrium binding equation which corrects for ligand depletion by the species being titrated [Segel (1975), eq II-54, p 74].

Competitive replacement of PLP from a preformed TS–PLP complex was typically accomplished as follows: A 1-cm path length cuvette containing 5.8 μM of TS monomer was made 7.9 μM in PLP, resulting in an increase of ~0.025 in A_{328} due to formation of the TS–PLP complex. dUMP or dTMP was added to reverse the absorbance change, and the decrease in A_{328} was plotted as a function of nucleotide concentration and fit to a modification of eq III-5 described by Segel [Segel (1975), p 105] to obtain binding constants for dUMP and dTMP.

Molecular Modeling. Modeling studies were performed using the Quanta (Polygen/Molecular Simulations, Waltham, MA) and MidasPlus (Ferrin et al., 1988) packages run on a Silicon Graphics Iris graphics workstation and used X-ray crystal structures of the neutral form of PLP-dihydrate (refcode: PLPHYD10) (Fujiwara, 1973) and dimeric TS bound to inorganic phosphate (Hardy et al., 1987; Finer-Moore et al., 1993). Throughout the study all atoms of TS were held fixed. We modeled the crystal structure of PLP-dihydrate into the active site of the *L. casei* TS structure by first superimposing the phosphate of PLP with the inorganic phosphate that is bound to TS in the crystal structure. Then, using the phosphorous as an origin of rotation, we manipulated the PLP-dihydrate to minimize the distance between the Cys 198 sulfur and the dihydrate oxygens; it was possible to virtually superimpose either oxygen with the Cys 198 sulfur. Complexes were then subjected to energy minimization using CHARMM (Brooks & Karplus, 1983)and the parameter set PARM30, as supplied in the Quanta package. Initial minimization used a steepest-descents algorithm to remove close contacts, and then subsequent minimization used the Adopted-Basis-Newton-Raphson (ABNR) algorithm.

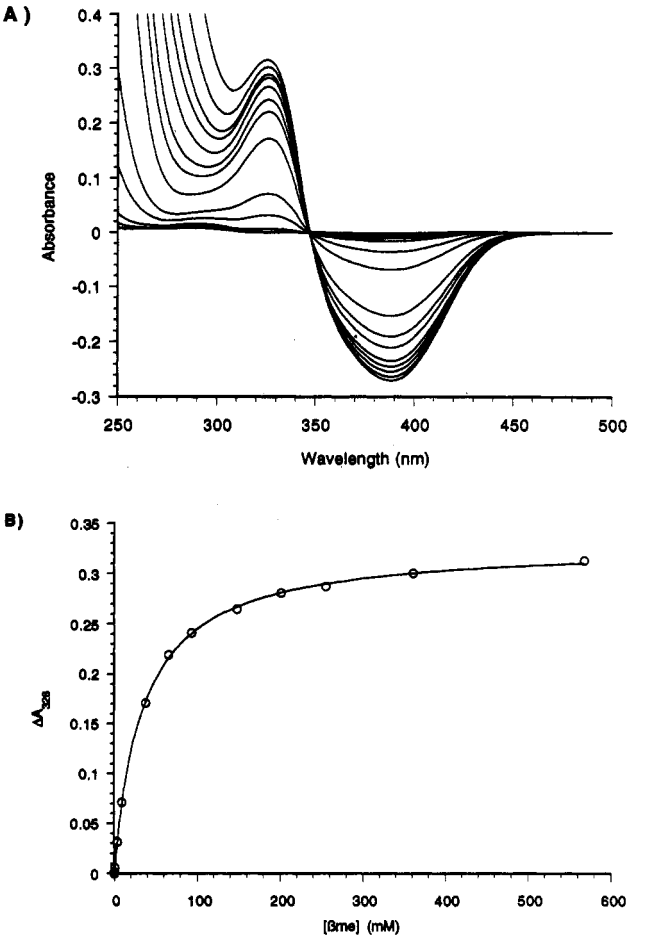


FIGURE 1: Interaction of PLP with βME. (A) UV/vis difference spectra of PLP + βME versus βME; the concentration of PLP was 59 μM, and concentrations of βME were varied from 0 to 568 mM. (B) Change of A_{328} versus βME concentration. Points are experimental, and the line is a best fit to eq II-54 from Segal (1975).

RESULTS

Interaction of PLP with Thiols and Amines. The reaction of thiols with PLP has been reported to give thiohemiacetals with absorbance maxima at 328 nm (Buell & Hansen, 1960); however, the reported data are insufficient to calculate equilibrium constants. The reactions between PLP and the thiols βME and DTT (thiols commonly used in the TS assays) were investigated by UV/vis difference spectroscopy. Figure 1A shows the UV/vis difference spectra of PLP titrated with

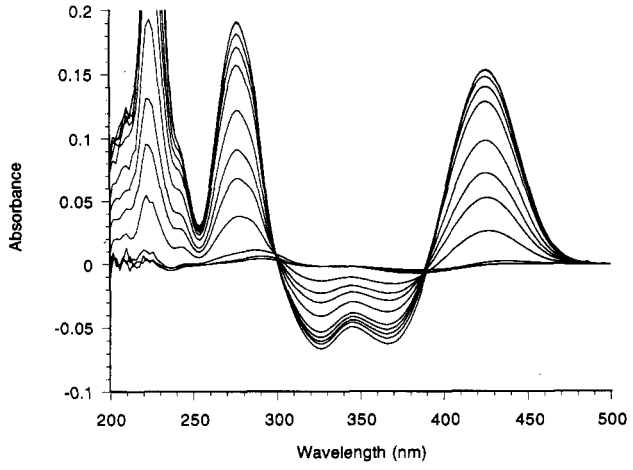


FIGURE 2: UV/vis difference spectra of 49 μM PLP + ε-aminocaproic acid versus ε-aminocaproic acid. The concentration of ε-aminocaproic acid was varied from 0 to 82 mM.

βME in the standard TES buffer. With increasing thiol concentrations, a differential absorbance maximum emerges at 328 nm ($\Delta\epsilon$ = 5460 M^{-1} cm^{-1}) due to thiohemiacetal formation, and a minimum appears at 388 nm ($\Delta\epsilon$ = –4838 M^{-1} cm^{-1}) due to the decrease of PLP aldehyde. Both of these changes saturate at high thiol concentration. The spectra of PLP in equilibrium with varying concentrations of βME show a well-defined isosbestic point at 347 nm, indicating the absence of detectable intermediates and side reactions. Figure 1B shows a fit of the data which provides a K_d value of 35 mM. Similar difference spectral titration of PLP with DTT shows λ_{max} =328 nm ($\Delta\epsilon$ = 5700 M^{-1} cm^{-1}), λ_{min} =388 nm ($\Delta\epsilon$ = –4860 M^{-1} cm^{-1}), and a single isosbestic point at 347 nm; the K_d of DTT was 13 mM or 26 mM per thiol group. NMR spectra of PLP in D_2O and TES buffer, pH = 7.4, showed that addition of 6–88 mM βME caused a titratable decrease in the aldehyde proton resonance (10.4 ppm) as expected with thiohemiacetal formation (K_d = 13 mM; data not shown).

Schiff base formation of PLP with α-amino acids has been extensively studied (Heinert and Martell, 1963; Kallen et al., 1985). We used UV/vis difference spectra of PLP with ε-aminocaproic acid as a model for the interaction of PLP with the ε-amino group of lysine residues. As shown in Figure 2, difference spectra show λ_{max} at 276 nm ($\Delta\epsilon$ = 3900 M^{-1} cm^{-1}) and 426 nm ($\Delta\epsilon$ = 3120 M^{-1} cm^{-1}), λ_{min} at 326 nm ($\Delta\epsilon$ = –1340 M^{-1} cm^{-1}) and 368 nm ($\Delta\epsilon$ = –1220 M^{-1} cm^{-1}), and isosbestic points at 300 and 389 nm. The K_d of the PLP–ε-aminocaproic acid adduct was 26 mM.

Interaction between PLP and TS. When increasing amounts of wild-type *L. casei* TS were added to a solution containing PLP, the UV/vis difference spectrum versus TS (method B) shows a λ_{max} of 328 nm and a λ_{min} of 388 nm, with an isosbestic point at 347 nm (Figure 3A). The decrease in absorbance at 388 nm is attributed to the loss of the aldehyde chromophore of PLP and the increase at 328 nm to thiohemiacetal formation. From spectra obtained at saturating concentrations of TS where all PLP should be bound to one subunit of the dimeric enzyme, extinction coefficients for bound PLP were calculated to be $\Delta\epsilon_{328nm}$ = 4670 M^{-1} cm^{-1} and $\Delta\epsilon_{388nm}$ = –4670 M^{-1} cm^{-1}.

Figure 3B shows the difference spectral titration of TS with PLP (method A). At concentrations of PLP up to about 25 μM, difference spectra show λ_{max} = 328 nm, λ_{min} = 388 nm, and an isosbestic point at 349 nm. At saturating concentrations of PLP, and assuming both sites of TS are occupied, we calculate $\Delta\epsilon_{328}$ = 5325 M^{-1} cm^{-1} and $\Delta\epsilon_{388}$ = –4935 M^{-1} cm^{-1} (both values are per monomer of TS). A plot of absorbance at 328 or 388 nm versus PLP concentration showed a K_d value

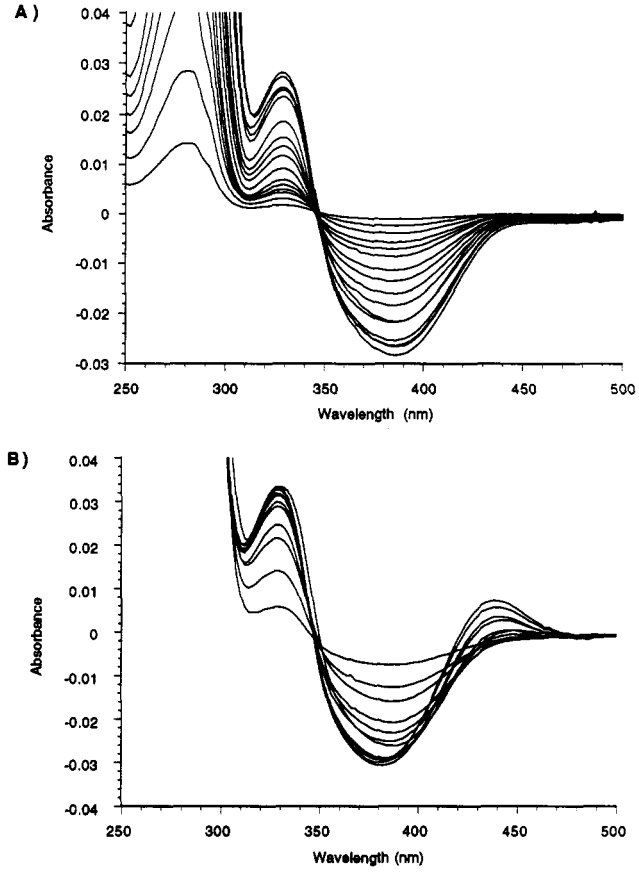


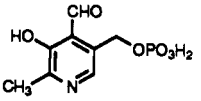
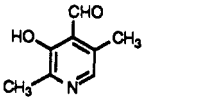
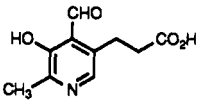
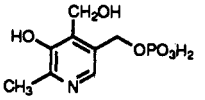
FIGURE 3: Interaction of PLP with TS. (A) UV/vis difference spectra of 5.5 μM PLP + TS versus TS; the concentrations of TS monomer were varied from 0 to 63 μM. (B) UV/vis difference spectra of 6.2 μM TS monomer + PLP versus PLP; the concentrations of PLP were varied from 0 to 26 μM.

of 0.5 ± 0.2 μM with 2 mol of PLP bound per mole of TS dimer. In these measurements, data were fit to an equation which compensates for ligand depletion by enzyme binding [Segel (1975), p74]. Nevertheless, because the measured absorbance changes were small and the experiments required TS concentrations significantly higher than the apparent K_d, there was a possibility for error in the calculation of free ligand. We therefore repeated the titration with PLP using a 10-cm path length cuvette and 0.6 μM TS; this gave a K_d of 0.8 μM, which is close to the TS concentration used and thus verified the original K_d value, the experimental approach, and the approach for fitting the data.

As the total concentration of PLP was increased above 20 μM, the isosbestic point was disrupted, and a new peak emerged with λ_{max} at 434 nm (Figure 3B). The latter is attributed to formation of a Schiff base between PLP and one or more lysine residues of TS. Using Schiff base formation between PLP and ε-aminocaproate as a model (K_d = 26 mM), we calculate that only about 0.03 mol of Schiff base is formed per mole of TS dimer over the range of PLP concentrations used to calculate K_d values; we therefore have not considered the possible effects of Schiff base formation on the interaction of interest.

Interaction between PLP and TS Mutants. We wished to unambiguously identify which of the two thiols of *L. casei* TS, Cys 198 or Cys 244, was responsible for the 330-nm absorbance of the TS–PLP complex. In the fully active C244T mutant, the only cysteine residue is the catalytic thiol Cys 198. Difference spectra of C244T TS plus PLP versus PLP obtained by method A are similar to those obtained with PLP and wild-type enzyme with λ_{max} = 328 nm and λ_{min} = 388 nm (data not shown). A K_d value of 0.8 ± 0.2 μM was obtained

Table I: Interaction of PLP and Analogs with *L. casei* Thymidylate Synthase

compound	structure	TS binding
PLP		K_d = 0.3 μM
5-deoxypyridoxal		K_d = 100 μM K_i = 60 μM
3-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)propionic acid		K_d = 60 μM
pyridoxol phosphate		K_i = 700 μM

from titration of the increase in absorbance at 328 nm, and at saturation, 2 mol of PLP was bound per mol of TS dimer. As with the wild-type enzyme, a red shift in the spectrum and a new peak at 434 nm are observed at higher concentrations of PLP (>20 μM) and are attributed to Schiff base formation.

In contrast, difference spectra of C198A TS plus PLP versus PLP showed no significant changes at low concentrations of PLP (<20 μM); in particular, there was no differential absorbance at 328 nm (data not shown). At higher concentrations, a peak at 434 nm appears and is attributed to Schiff base formation between PLP and enzyme amino groups. Similar results were obtained with the C198S mutant.

Interaction of PLP Analogs with TS. A summary of TS binding by the PLP analogs investigated appears in Table I. The phosphate of PLP has been implicated as an important site of interaction with TS because pyridoxal, which lacks the phosphate moiety of PLP, is a poor inhibitor of TS (Chen et al., 1989). We also found that treatment of TS with pyridoxal (to 200 μM) did not give the characteristic thiohemiacetal absorbance seen with PLP. However, since pyridoxal exists as a stable intramolecular hemiacetal in solution (Nakamoto & Martell, 1959), its ability to form an intermolecular thiohemiacetal with TS is compromised and the analog is not a valid test of the importance of the phosphate group in the interaction. Thus, we examined the interaction of 5-deoxypyridoxal, which is missing the hydroxyl group of pyridoxal and thus retains the aldehyde moiety in aqueous solution (Metzler & Snell, 1955). The difference spectra of wild-type TS plus 5-deoxypyridoxal versus 5-deoxypyridoxal (method A) showed no change at ligand concentrations up to 20 μM. As the concentration of 5-deoxypyridoxal was increased to 500 μM, complex changes in the difference spectra occurred which suggested both thiohemiacetal and Schiff base formation ($\Delta\lambda_{max}$ = 328 and 434 nm, respectively). Titration of absorbance at 328 nm occurred with a K_d of about 100 μM, wheras inhibition of TS-catalyzed dTMP formation gave a K_i of 60 μM. We also have studied the binding of TS to the analog 3-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)propionic acid, where the phosphate of PLP is substituted by a carboxylate. Addition of this analog to TS gave spectral changes similar to those induced by PLP, and titration of the 328-nm chromophore indicated a K_d of 60 μM, similar to the K_i obtained for the inhibition of TS by 5-deoxypyridoxal. Thus, it appears that the phosphate moiety of PLP provides about 2.8 kcal mol^{-1} of binding energy to the TS–PLP complex.

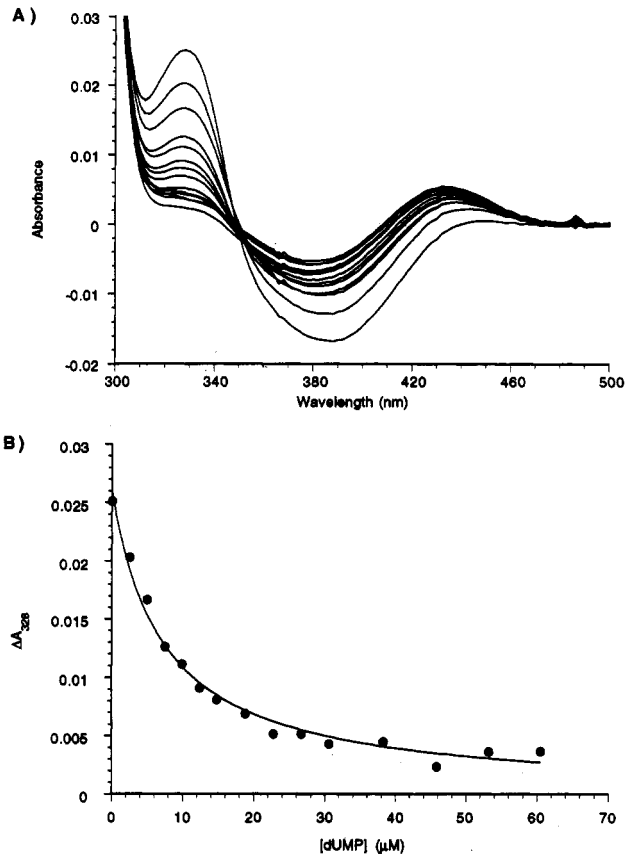


FIGURE 4: Interaction of dUMP with PLP-TS complex. (A) UV/vis difference spectra of PLP-TS complex. The concentration of TS monomer was 4.4 μM, the concentration of PLP was 8.2 μM, and the concentrations of dUMP were varied from 0 to 60 μM. (B) Replot of the change of A_{328} versus dUMP concentration. Points are experimental, and the line is a least-squares fit to eq III-5 from Segel (1975).

We wished to assess the importance of thiohemiacetal formation for formation of the TS–PLP complex. Pyridoxamine phosphate is a poor inhibitor of TS (Chen et al., 1989); however, this may be due to untoward electrostatic interactions between the enzyme and the protonated amino group (pK_a = 10.7; Metzler et al., 1973). We therefore reduced the aldehyde of PLP to obtain the alcohol analog, pyridoxol phosphate, which is isosteric with pyridoxamine phosphate. This analog bound about 1400-fold poorer than PLP to TS as determined by its ability to inhibit the formation of dTMP (K_i =700 μM). Thus, the formation of the thiohemiacetal provides about 4.3 kcal mol^{-1} of binding energy to the TS–PLP complex.

To investigate the contribution of the pyridine nitrogen of PLP to TS binding, we examined *N*-methyl-PLP and found that it induced spectral changes similar to those induced by PLP, and bound to TS with an approximate K_d of 1 μM.

Competition of PLP Binding by dUMP and dTMP. Figure 4A shows that upon titration of the preformed PLP-TS complex with dUMP the differential absorbance at 328 and 388 nm is completely lost. This indicates loss of the thiohemiacetal and reappearance of the aldehyde carbonyl upon displacement of PLP by dUMP from the TS–PLP complex. Figure 4B shows a replot of A_{328} as a function of added dUMP, which yielded a K_d of 0.5 μM for dUMP; this value is in agreement with the K_{ia} determined from steady-state kinetics, but some 10-fold lower than K_m for dTMP formation (Daron & Aull, 1978). Also, since 2 mol of PLP is bound per mole of TS dimer, the complete displacement of PLP by dUMP is consistent with a model in which 2 binding sites per dimeric TS are occupied by dUMP in the absence

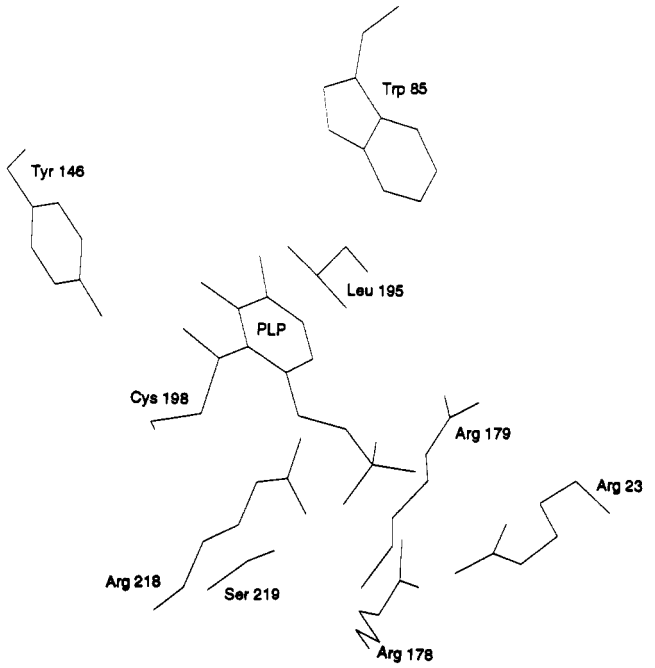


FIGURE 5: Energy-minimized model structure of the TS–PLP adduct. The *R* configuration of the PLP thiohemiacetal is shown.

of CH_2H_4folate; however, we cannot rule out the possibility that binding of 1 mol of dUMP/mol of TS dimer displaces PLP from both active sites. A similar experiment was performed using dTMP and indicated that 2 molecules of dTMP are bound by the TS dimer with a K_d of 1.6 μM.

Molecular Modeling of the TS–PLP Complex. The initial energy minimization of the *R* and S forms of the TS–PLP complex resulted in similar conformations of the PLP adduct (Figure 5). In the modeled structure, a thiohemiacetal has been formed between Cys 198 and the PLP aldehyde, and the PLP phosphate is liganded by Arg 23, Arg 218, Arg 178, and Arg 179 in a manner similar to that observed for the dUMP phosphate in the TS–dUMP crystal structure (Finer-Moore, 1993). Also within 3.5 Å of any atom of PLP were Trp 85, Tyr 146, Leu 195, and Ser 219. It appeared that the side-chain methyl groups of Leu 195 could interact with the π-electron system of the pyridoxal ring. In the modeled structure of the *R* adduct, the hydroxyl of Tyr 146 was within 3.6 Å of the thiohemiacetal hydroxyl group. With a small movement, it is possible that these could form a hydrogen bond. No other obvious interactions between PLP and TS were apparent.

DISCUSSION

Molecular Modeling. It has been proposed that the inhibition of TS by PLP results from formation of a TS–PLP complex in which the monophosphate ester of PLP binds to the phosphate binding site of TS, and the aldehyde moiety forms a thiohemiacetal to the active site thiol of *L. casei* TS (Chen et al., 1989). We investigated whether the putative TS–PLP adduct was compatible with the three-dimensional structure of TS and, if so, what other TS–PLP interactions might be feasible. We modeled the crystal structure of PLP-hydrate into the crystal structure of the active site of *L. casei* TS. When the phosphate moiety of PLP-hydrate was superimposed with the phosphate in the TS–P_i structure, a thiohemiacetal could be formed between the PLP aldehyde carbon and the thiol of Cys 198 without obvious unfavorable interactions between the remainder of PLP and the enzyme. At this level of modeling, there was a good fit with either the *R* or *S* configuration of the putative TS–PLP thiohemiacetal adduct.

Next, we investigated the range of conformational freedom available to PLP with the constraints of a rigid protein, phosphate binding by Arg 23, 178, 179, and 218, and thiohemiacetal formation with Cys 198. Thus, both the *R* and *S* configurations of the PLP tethered to a rigid TS were subjected to energy minimization. The resulting models placed the PLP in proximity of several side chains of the protein, in particular, Trp 85, Tyr 146, Leu 195, and Ser 219. With the exception of Trp 85, which is substituted by asparagine in some species, all of these residues are highly conserved in TS [see Perry et al. (1990)]. Further modeling studies were not pursued, and efforts were directed toward determination of the crystal structure of the complex (in progress).

Model Studies. Reaction of PLP with thiols results in reversible formation of the corresponding thiohemiacetals with apparent K_d values of ~30 mM. The adducts show UV/vis absorbance difference spectra with a maximum at 328 nm characteristic of the thiohemiacetal, and a minimum at 388 nm which results from loss of absorbance of the aldehyde carbonyl group of PLP. Reaction of PLP with ϵ-aminocaproic acid was used as a model for the reaction of PLP with the ϵ-amino group of lysine. This reaction caused a differential absorbance peak at 426 nm which could be titrated (K_d = 26 mM), as well as changes at other wavelengths. The spectra of these model systems were used to analyze the interaction of PLP with TS.

TS–PLP Interactions. The reaction of PLP with TS at pH 7.4 results in formation of a new chromophore with a λ_{max} at 328, indicative of a thiohemiacetal, and a decrease in A_{388} indicating loss of the PLP aldehyde carbonyl. The $\Delta\epsilon$ at both wavelengths is approximately 5000 M^{-1} cm^{-1}, which is very similar to the $\Delta\epsilon$ observed for model reaction of thiols with PLP; also, the amount of thiohemiacetal generated corresponds to the amount of PLP reacted. The K_d of the TS–PLP complex is 0.5 μM, which is similar to the K_i of PLP as an inhibitor of TS (Chen et al., 1989). At higher concentrations of PLP, a high-wavelength peak appears at 434 nm which suggests Schiff base formation with primary amines of the protein such as the ϵ-amino group of lysine residues. However, the K_d for Schiff base formation is high, and we calculate that only about 3% of TS is involved in Schiff base formation at concentrations of PLP required to saturate the high-affinity site.

There are two cysteine residues in *L. casei* TS which could, in principle, give rise to the thiohemiacetal absorbance: Cys 198, which is the catalytic nucleophile, and Cys 244, which is remote from the active site. Treatment of the C244T mutant, which is as active as wild-type TS, with PLP results in a UV/vis difference spectrum that is almost identical to that of the wild-type TS–PLP complex, with the similar K_d of 0.8 μM. Thus, Cys 244 of TS is not responsible for the thiohemiacetal absorbance at 328 nm. In contrast, the inactive C198A and C198S mutants do not show the peak at 328 nm when treated with PLP, but do show the changes associated with Schiff base formation at high PLP concentrations. We conclude that Cys 198 of TS is solely responsible for the 328-nm absorbance assigned to thiohemiacetal formation with PLP and is associated with high-affinity binding.

Evidence that further supports the idea that PLP is bound to the active site of TS are the observations that PLP is a competitive inhibitor of TS with respect to dUMP (Chen et al., 1989) and, as shown here, that PLP can be completely displaced from TS by either dUMP or dTMP. When the TS–PLP complex is treated with the substrate dUMP, there

is a concentration-dependent loss of absorbance at 328 nm, which provides a K_d for dUMP of 0.3 μM. This is in agreement with the value obtained by steady-state kinetics (Daron & Aull, 1978), and by titration of dUMP-induced changes in the circular dichroism spectrum of TS (Leary et al., 1975), but about 10-fold lower than that obtained by equilibrium dialysis (Galivan et al., 1976); we do not know the reason for this discrepancy. Further, the differential absorbance of the TS–PLP complex is completely lost with saturating dUMP, showing that the substrate displaces both molecules of PLP from the dimeric enzyme. The TS–PLP complex can likewise be completely displaced with dTMP. In this laboratory, displacement of the TS–PLP complex serves as a convenient spectrophotometric assay for competitive binding of ligands which occupy the nucleotide binding site of TS (Liu & Santi, 1993).

Importance of PLP Functional Groups for TS Binding. In accord with the putative importance of thiohemiacetal formation in the TS–PLP complex, reduction of the aldehyde moiety of PLP to the alcohol, giving pyridoxol phosphate, resulted in a decrease in binding of about 1400-fold, or about 4.3 kcal mol^{-1}. The importance of the phosphate ester of PLP in binding to TS was demonstrated by showing that 5-deoxypyridoxal (K_i = 60 μM) and 3-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)propionic acid (which substitutes a carboxylate for the PLP phosphate and binds to TS with a K_d of 60 μM) are ~120-fold poorer inhibitors than PLP. Thus, about 2.8 kcal mol^{-1} in binding energy can be attributed to interactions between the enzyme and the phosphate of PLP. Summation of the binding energies attributed to enzyme–phosphate interactions and thiohemiacetal formation predicts a K_d for the TS–PLP adduct of ~6 μM, 10-fold higher than the experimentally observed value for PLP, and suggests that ~1.4 kcal mol^{-1} of binding energy arises either from other enzyme–PLP interactions or from the reduced entropy of having both a phosphate and an aldehyde moiety on the same molecule.

Implications for Drug Design. A knowledge of the interaction of PLP with TS may provide insights into approaches for the design of a new class of inhibitors which do not resemble the substrates of the enzyme. It is clear that the phosphate and aldehyde moieties of PLP are important to binding. Unfortunately, the necessity for the phosphate group may be problematic in drug design, since phosphate monoesters do not readily penetrate cells. To overcome this, it would be necessary to (a) design a prodrug analog of PLP which is converted to a phosphate *in vivo*, (b) find a mimic for the phosphate group, or (c) enhance binding elsewhere on the molecule so the phosphate can be omitted. In contrast, although formation of the reversible thiohemiacetal between PLP and TS is crucial for high affinity, even more favorable covalent bonds can be envisioned with analogs containing carbonyl groups with greater propensity for covalent bond formation with Cys 198. For example, this might be achieved with electron-withdrawing substituents on the carbonyl carbon or pyridine ring. We do not know whether the pyridine ring of PLP is important for binding to TS, although we think it plays some role. An understanding of the interaction of the PLP pyridine moiety with the enzyme may permit design of even more potent inhibitors of TS, either by optimizing favorable interactions or by using the heterocycle as a scaffold for other groups which may interact favorably with the enzyme. Toward this end, efforts are being directed toward solution of the X-ray structure of the TS–PLP complex.

Finally, an issue not addressed in this work which must be considered is whether there is any physiological relevance to the binding of PLP to TS.

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