Stereoelectronic Activation of Methylenetetrahydrofolate by Thymidylate Synthase: Resonance Raman Spectroscopic Evidence†

J. C. Austin,‡,§ A. Fitzhugh,|| J. E. Villafranca,⊥ and T. G. Spiro*,‡

Department of Chemistry, Princeton University, Princeton, New Jersey 08544, Agouron Pharmaceuticals Inc., 3565 General Atomics Court, San Diego, California, 92121, and PRI/DynCorp, National Cancer Institute−FCRDC, P.O. Box B, Frederick, Maryland 21702

Received September 29, 1994; Revised Manuscript Received March 27, 1995⊗

ABSTRACT: Resonance Raman (RR) spectra are reported for the ternary complex of *Escherichia coli* thymidylate synthase with the cofactor 5,10-methylenetetrahydrofolate (CH_2-H_4-folate) and the inhibitor 5-fluoro-2′-deoxyuridylate, excited at 337 or 356 nm, in resonance with perturbed absorption bands of the *p*-aminobenzoylglutamate (PABA-Glu) portion of the cofactor. For comparison, RR spectra were obtained with 260 nm excitation for PABA-Glu in various solvents, and for CH_2H_4-folate and H_4-folate in aqueous solution. These reference spectra are assigned to modes of PABA-Glu in its benzenoid form. The ternary complex RR spectra are very different, however, and are assigned, with the aid of isotopic data, to the PABA-Glu in a predominantly quinoid form. Similar spectra were obtained for the ternary complexes of the E58Q and K48Q mutants, indicating that neither Glu58 nor Lys48 are essential for maintaining the quinoid structure, even though their side chains complement the dipolar charge distribution of the quinoid form of PABA-Glu. Since these are the only charged residues in the PABA-Glu vicinity, electrostatic stabilization is not essential to maintenance of the quinoid structure. It is proposed that quinoid formation results from steric forces, probably resulting from the protein conformation change known to accompany cofactor binding, which enforce coplanarity of the PABA-Glu ring and substituents. This stereoelectronic change activates the cofactor by opening the methylene bridge. A second RR spectrum of the ternary complex, previously proposed to reflect an alternate structure, is shown to result instead from irreversible formation of a laser-induced photoproduct.

Thymidylate synthase (TS) catalyzes the methylation of 2′-deoxyuridine 5′-monophosphate (dUMP) to 2′-deoxythymidine 5′-monophosphate (dTMP), using the cofactor 5,10-methylenetetrahydrofolate (CH_2H_4-folate) as the carbon donor (Santi & Danenberg, 1984). The cofactor CH_2H_4-folate is oxidized to dihydrofolate in the reaction. The details of the TS mechanism have been of particular interest due to the discovery of a range of anti-cancer drugs which act by forming covalent inhibitor complexes with TS (Santi & Danenberg, 1984; Cisneros et al., 1988). For example, 5-fluoro-2′-deoxyuridylate (FdUMP), a metabolite of the antipyrimidines 5-fluorouracil and 5-fluorodeoxyuridine, is known to form a covalent ternary complex with TS (Danenberg et al., 1974; Santi et al., 1974; James et al., 1976). It has been proposed for many years (Friedkin, 1959) that a key intermediate in the TS mechanism is the covalent ternary complex, which contains a covalent bond between a cysteine sulfur and C_6 of dUMP and a covalent bridge between C_5 of dUMP and N_5 of the cofactor. Several studies have focused on the structural characterization of analogs of the Friedkin intermediate, and in particular the ternary complex of TS, FdUMP, and CH_2H_4-folate (Figure 1) (Matthews et al., 1990; Fitzhugh et al., 1986; James et al., 1976; Santi et al., 1974; Donato et al., 1976). The structure of the ternary complex shown in Figure 1 is assumed to be stable because F^- is a poor leaving group and cannot be extracted by the enzyme to form the reaction products.

The crystal structures of TS ternary complexes including the TS•FdUMP•CH_2H_4-folate ternary complex have provided valuable insights into the mechanism of TS and the residues that are probably important for binding and catalysis (Finer-Moore et al., 1990; Matthews et al., 1990). In addition, the binding affinities of certain types of antifolate drugs have yielded information on the steric and electronic requirements in the TS active site (McNamara et al., 1990; Varney et al., 1992). It is clear from several spectroscopic studies that there is a significant perturbation of the *p*-aminobenzoylglutamate (PABA-Glu) group of the cofactor on forming the TS ternary complex with FdUMP (Fitzhugh et al., 1986; Donato et al., 1976). The spectroscopic perturbation could be related to either a conformational change in the cofactor produced by steric restrictions imposed by the enzyme, or by the polarity of the enzyme binding site. A full understanding of the nature of the spectroscopic perturbation and its likely cause may provide vital information on the requirements for effective binding of inhibitors.

RR spectra have been obtained previously for the ternary complex of *Lactobacillus casei* TS with FdUMP and CH_2H_4-folate (Fitzhugh et al., 1986). The RR study confirmed that the PABA-Glu group of the cofactor is responsible for the

† This work was supported in part by NIH Grant GM25158 (to T.G.S.) and by Federal Funds from the Department of Health and Human Services (to A.F.).

* Author to whom all correspondence should be addressed.

‡ Princeton University.

§ Present address: Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461.

|| PRI/Dyn Corp.

⊥ Agouron Pharmaceuticals.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1995.

0006-2960/95/0434-7678$09.00/0

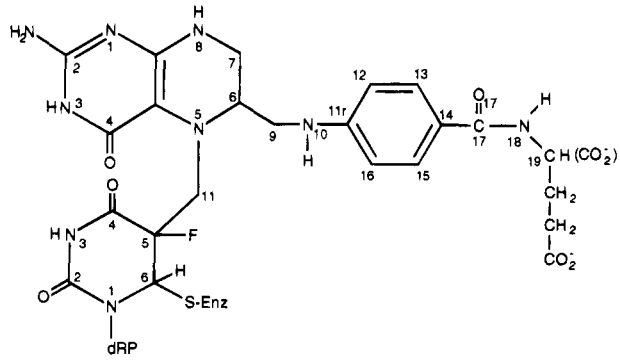

FIGURE 1: Structure and numbering of the TS•FdUMP•CH_2H_4-folate ternary complex. dRP = 2′-deoxyribose 5′-monophosphate.

322-nm absorption of the ternary complex. It was also suggested in the previous RR study that an alternative structure to the Friedkin intermediate analog (Figure 1) coexisted in the TS ternary complex. In the present study, it is shown that there is no "alternative structure" and that the previous assignment arose from the formation of a photoproduct species. In addition, mutants of TS are studied in conjunction with model compounds to identify the cause of the unusual spectroscopic perturbation of the PABA-Glu group in the TS ternary complex.

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Complex Formation. TS and the TS mutants were isolated from *E. coli* as described by Matthews et al. (1990) and stored in 30%, v/v, glycerol solutions at −80 °C. For formation of the ternary complexes, TS was dialyzed against pH 7 buffer containing 50 mM potassium phosphate (or 10 mM Tris), 1 mM EDTA, 25 mM KCl, and 75 mM β-mercaptoethanol. For preparations in D_2O, dialyzed enzyme was diluted into D_2O buffer and reconcentrated. Enzyme concentrations were estimated using an estimated ϵ_{280} of 9.5×10^4 M^{-1} cm^{-1}, which was calculated from the extinction coefficient of the *L. casei* enzyme (Donato et al., 1976) by scaling to account for the different number of aromatic amino acid residues. 5,10-Methylenetetrahydrofolate (CH_2H_4-folate) was prepared by adding tetrahydrofolate (Calbiochem) to a solution containing 0.05 M $NaHCO_3$, 0.07 M formaldehyde, and 0.25 M β-mercaptoethanol or 10 mM dithiothreitol (Osborn et al., 1960). The formation of CH_2H_4-folate was verified by the shift in the absorption maximum from 298 nm (tetrahydrofolate, H_4-folate) to 294 nm (CH_2H_4-folate).

For ternary complex formation, a 5−10-fold excess of FdUMP (Sigma) was added to the enzyme, followed by addition of a 30−50-fold excess of CH_2H_4-folate or H_4-folate. The mixture was allowed to sit at room temperature for 10 min, but at all other times it was maintained at 4 °C. The ternary complex was separated from free cofactor and FdUMP by column chromatography (G-25) in all cases except preparations in D_2O. For complexes formed in D_2O solutions, several dilution-reconcentration steps using Centricon-10 concentrators were necessary to remove unbound cofactor, which produced sufficient fluorescence to swamp the Raman signal.

The absorption ratio A_{280}:A_{322} was used as a guide to the extent of complex formation. For *L. casei* complexes, this ratio has been carefully quantitated in terms of the amount of 2:2:1 (both sites complexed) and 1:1:1 complexes (Donato et al., 1976). For *E. coli*, where the enzyme absorbance at 280 nm is slightly different, an accurate calibration of the A_{280}:A_{322} ratio has not been performed. However, by assuming the same extinction coefficient for the bound cofactor (ϵ_{322}), and by scaling the enzyme ϵ_{280} values, an approximate calibration was obtained. Typically, WT complexes showed A_{280}:A_{322} ratios of 2.3−2.5:1 (see Figure 2a), which indicated >75% formation of the 2:2:1 ternary complex (the remainder is 1:1:1 complex). The K48Q mutant formed complexes with A_{280}:A_{322} ratios of *ca.* 3:1, indicating only *ca.* 40% 2:2:1 ternary complex (the remainder is 1:1:1 complex). The E58Q mutant formed the 2:2:1 complex even less readily, having A_{280}:A_{322} ratios in the range 3.4−4 (between 0 and 15% 2:2:1 complex; the remainder is 1:1:1 complex and uncomplexed TS). All UV−vis spectra were obtained using a Hewlett-Packard 8451A diode array spectrophotometer.

Isotopomers. $^{13}C_{11}$-labeled CH_2H_4-folate was prepared as described above, using ^{13}C-labeled formaldehyde. $^{13}C_{17}$-Labeled CH_2H_4-folate was prepared by enzymatic reduction of pteroylglutamic acid [^{13}C]benzoyl carbonyl (Plante et al., 1980) using dihydrofolate reductase from *L. casei* . The required 4-aminobenzoic acid [^{13}C]benzoyl carbonyl was prepared by reducing 4-nitrobenzoic acid [^{13}C]benzoyl carbonyl (99% enrichment; Cambridge Isotope Labs) with H_2 over Raney nickel. (C_{12},C_{16})2D-labeled CH_2H_4-folate was similarly prepared from pteroylglutamic acid, whose benzoyl ring was preferentially deuterated at the C_{12} and C_{16} positions using the method of Hackey et al. (1978). Mass spectral and NMR analyses showed that the product contained at least 93% of the 2D species.

Raman Spectroscopy of Ternary Complexes. For experiments undertaken with 337-nm excitation, samples (0.3−0.5 mL) were concentrated to ca. 0.15−0.3 mM. For experiments using 356 or 363-nm excitation, samples were concentrated to 0.3−0.5 mM. Samples were maintained between 4 and 10 °C by blowing cold N_2 on the sample cuvette. Samples were irradiated with 5−15 mW of 337.5- or 356.4-nm excitation from a Kr^+ laser or with 363-nm excitation from an Ar^+ laser. Raman scattering was dispersed using a Spex Triplemate spectrometer equipped with 1200 grooves/mm filter stage gratings and a 2400 grooves/mm spectrograph stage grating. The spectral resolution in the experiments was *ca.* 8 cm^{-1}. Raman scattered light was detected using a cooled (−30 °C) diode array detector (EG&G PAR). Spectra were generally accumulated for 10−20 min at 337 nm and for 30−40 min at 356 or 363 nm. After 40 min of 356- or 363-nm excitation at >10 mW, a significant amount of photoproduct could be detected in both the Raman and absorption spectra (see Results and Discussion). The laser power and irradiation times employed with 337-nm excitation were generally not sufficient to observe significant photoproduct formation, although it does occur at this wavelength. Spectra were calibrated using standard wavenumber values for indene, ethanol, and dimethylformamide, giving a wavenumber accuracy of 1−1.5 cm^{-1}.

Model Compound Studies. Solutions of *p*-aminobenzoylglutamic acid (Sigma), H_4-folate, and CH_2H_4-folate were irradiated with 0.3-mW 260-nm excitation at 250 Hz. The 260-nm radiation was produced by frequency doubling the output of an excimer-pumped dye laser (Lambda Physik FL3002). The Raman scattering was dispersed in a 1.25 m

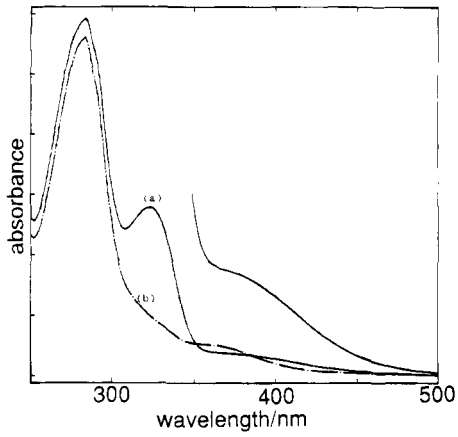

FIGURE 2: Absorption spectra of TS ternary complex with FdUMP and CH_2H_4-folate (a) before UV irradiation and (b) after prolonged UV irradiation.

single grating monochromator (Spex 1269) and detected with a cooled diode array detector (Princeton Instruments), as described previously (Austin et al., 1992).

RESULTS

(1) The Ternary Complex Forms a Photoproduct. In our previous RR study of the TSase ternary complex, different RR bands were observed with excitation at 337 and 356 nm (Fitzhugh et al., 1986). It was suggested that two different structures coexist at the active site and give rise to the two long-wavelength UV absorption bands, at 322 and 380 nm (Figure 2). The different excitation wavelengths were thought to produce selective enhancements for the two different chromophores. We now find that the RR bands previously reported at 337 nm are also observed with 356-nm excitation, provided the exposure to the laser beam is kept to a minimum, as seen in Figure 3. With prolonged 356-nm laser exposure, new bands gradually grow in at 1430, 1459, 1488, and 1656 cm^{-1}, the same bands reported previously at this wavelength, at the expense of the original spectrum. Evidently, a photoproduct is formed via irradiation of the ternary complex. No attempt was made to exclude O_2 from the solutions, and it is not known whether the photoproduct generation is O_2 dependent.

The RR spectrum of the photoproduct, obtained by subtraction of the short exposure from the long exposure spectrum, is shown at the bottom of Figure 3. The absorption spectrum also undergoes a drastic change upon prolonged exposure to the laser beam. As shown in Figure 2, the 322 and 370-nm absorption bands of the ternary complex disappear, and a new weak band is seen at 360 nm. This new band is no doubt responsible for the enhancement of the photoproduct RR spectrum.

We are uncertain about the nature of the photoproduct. In contrast to the ternary complex bands (see below), none of the photoproduct RR bands show any sensitivity to isotope substitution of the PABA portion of the cofactor. Thus, an alteration of the pterin ring seems likely, and a polycyclic chromophore would be consistent with the multiplicity of vibrational bands above 1300 cm^{-1}. The same RR spectrum was obtained whether CH_2H_4-folate or H_4-folate was used in preparing the ternary complex, implying that if the new chromophore is a modified pterin, the bond from N_5 to the uracil ring (Figure 1) is not intact.

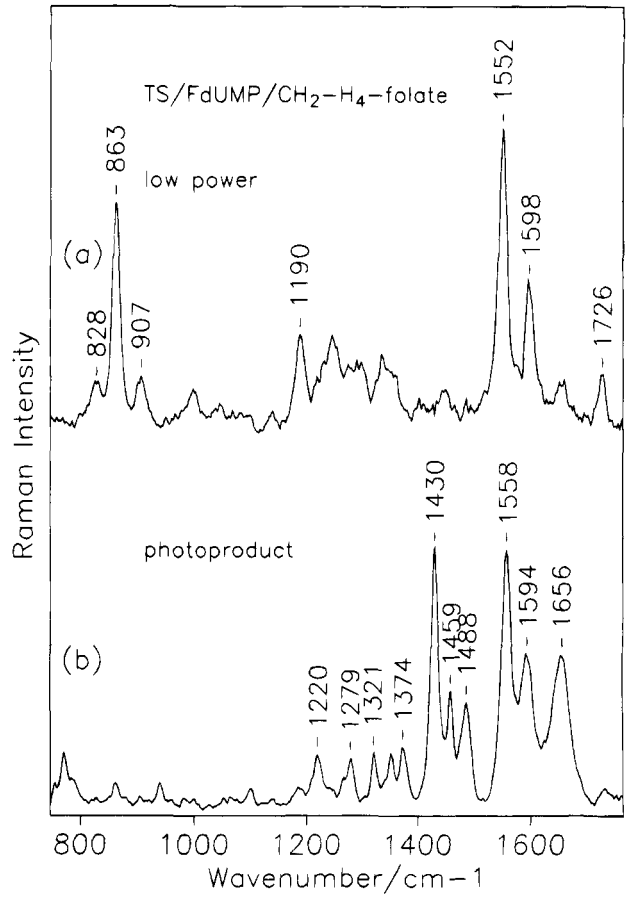

FIGURE 3: 356-nm-excited RR spectra of the TS·FdUMP·CH_2H_4-folate ternary complex: (a) short exposure times; (b) difference spectrum of long exposure minus short exposure, to reveal the photoproduct RR spectrum.

(2) Vibrational Assignments for the Ternary Complex and Model Compounds. (A) PABA-Glu and Folate. To interpret the ternary complex RR spectrum, we examined spectra for CH_2H_4-folate, as well as H_4-folate, in H_2O and D_2O (Figure 4), and for PABA-Glu in various solvents (Figure 5). The excitation was at 260 nm, near resonance with the PABA absorption band at 274 nm in H_2O. The RR band frequencies and suggested assignments are given in Table 1. UVRR spectra of substituted benzene molecules characteristically show a pair of ring modes, ν_{8a} and ν_{8b}, near 1600 cm^{-1} (Dollish et al., 1974). The higher frequency band, ν_{8a}, is strong, while ν_{8b} is weaker, and ν_{8b}, but not ν_{8a}, is sensitive to substituent interactions. This pattern is evident in the PABA-Glu spectra, which are dominated by the invariant 1608 cm^{-1} ν_{8a} band. This band is seen as well in the CH_2H_4-folate and H_4-folate spectra, which resemble the PABA-Glu spectra closely, consistent with RR enhancement exclusively of the PABA-Glu fragment in the folates.

The weaker band at 1574 cm^{-1} in PABA-Glu, assigned to ν_{8b}, shifts 10 cm^{-1} in D_2O, due to H/D exchange at the amine and amide substituents. This sensitivity is largely associated with exchange at the amine rather than the amide group, as can be seen from the spectrum of CH_2H_4-folate, which lacks an amine proton; the D_2O shift is only 2 cm^{-1} in this case. Moreover, the ν_{8b} frequency is the same as that observed for H_4-folate in D_2O, 1558 cm^{-1}. It is evident that coupling with the amine N−H bend forces the H_4-folate ν_{8b} frequency up to 1576 cm^{-1}, an effect prevented in

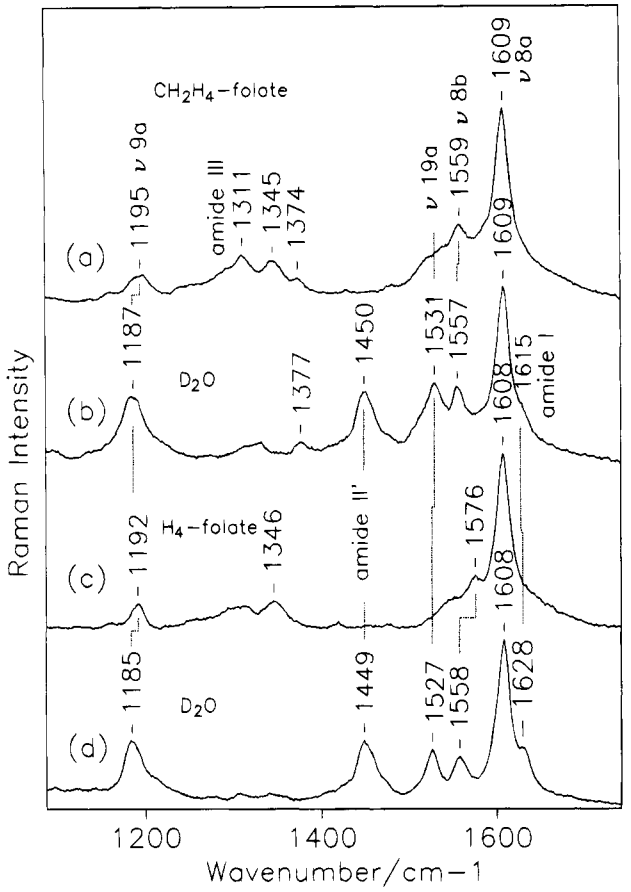

FIGURE 4: 260-nm-excited RR spectra of (a) CH_2H_4-folate in H_2O, (b) CH_2H_4-folate in D_2O, (c) H_4-folate in H_2O, and (d) H_4-folate in D_2O.

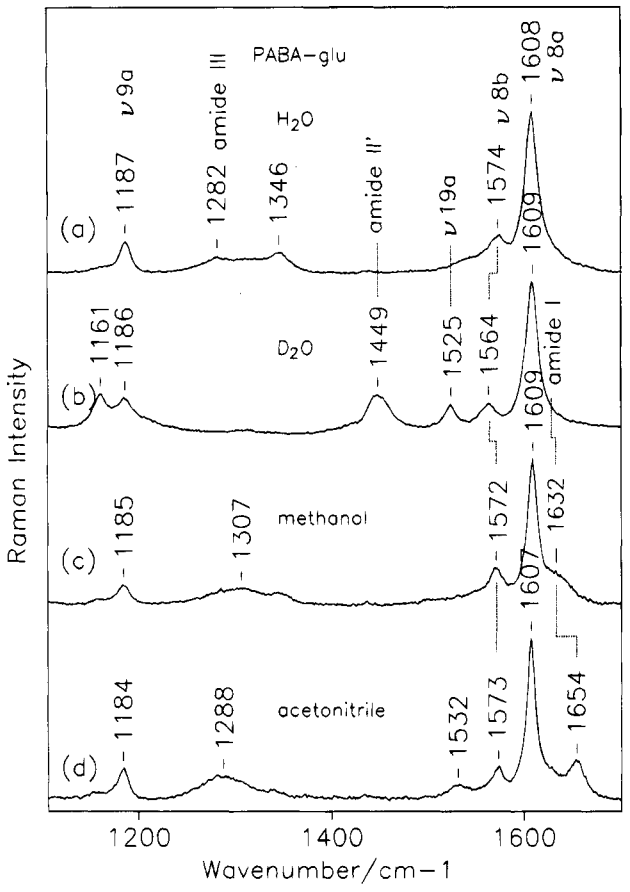

FIGURE 5: 260-nm-excited RR spectra of PABA-Glu in (a) H_2O, (b) D_2O, (c) MeOH, and (d) acetonitrile.

CH_2H_4-folate by the replacement of the N−H bond with an N−C bond.

Other ring modes can be identified: ν_{19a} at 1525−1532 cm^{-1}, ν_{9a} at 1185−1195 cm^{-1}, and the breathing mode, ν_1, at 850−870 cm^{-1} (spectra not shown in this region; see Table 1 for frequencies). All of these are somewhat sensitive to D_2O, indicating pervasive coupling of the ring modes with the amine and amide substituents.

The classical modes of the amide group, amide I, II and III (Dollish et al., 1974) are also detectable in the spectra. Amide I, the carbonyl stretch, is expected to be above 1600 cm^{-1} and is clearly seen at 1654 cm^{-1} for PABA-Glu dissolved in acetonitrile (Figure 5, bottom). It becomes a 1632-cm^{-1} shoulder in methanol and shifts under the strong ν_{8a} band in H_2O. The amide I frequency is known to be lowered by H-bond donation to the carbonyl O atom. The prominence of the band in acetonitrile implies that the amide group, which is conjugated to the ring, participates significantly in the resonant $\pi-\pi^*$ transition associated with the PABA-Glu 274-nm absorption band. CH_2H_4-folate and H_4-folate exhibit shoulders at 1615 and 1628 cm^{-1} in D_2O (Figure 4), which is probably amide I. The shoulder is obscured in H_2O by a wing on the ν_{8a} band, which arises from the broad H_2O bending mode at 1640 cm^{-1}.

Amide II and III, expected at about 1550 and 1300 cm^{-1}, are mixtures of C−N stretching and N−H bending coordinates. Upon N−H/D exchange, they collapse to a single strong band at about 1450 cm^{-1}, called amide II′, which is essentially a pure C−N stretching. Amide II′ is seen clearly in all the D_2O spectra, at 1449 cm^{-1}. Amide III candidate bands are seen weakly in the H_2O solutions, at 1282 cm^{-1} for PABA-Glu and at 1311 cm^{-1} for the folates. Amide II is obscured by overlap with the ν_{19a} band at 1525−1530 cm^{-1}, but it is seen to fill in the valley between ν_{19a} and ν_{8b}. Its disappearance in D_2O leaves both ν_{19a} and ν_{8b} more sharply defined. There is an additional D_2O-sensitive band at 1345 cm^{-1}, which is currently unassigned, and CH_2H_4-folate has a band at 1374 cm^{-1}, which is weakly sensitive to D_2O and is not present in H_4-folate; it may be associated with the extra N−C bond in the former. PABA-Glu has an extra band at 1161 cm^{-1}, which appears only in D_2O.

Table 1: RR Band Wavenumbers (cm^{-1}) and Assignments for PABA-Glu in Various Solvents, and for Tetrahydrofolate (H_4-folate) and Methylenetetrahydrofolate (CH_2H_4-folate) in H_2O and D_2O

PABA-Glu				H_4-folate		CH_2H_4-folate		
H_2O	D_2O	MeOH	acetonitrile	H_2O	D_2O	H_2O	D_2O	assignment
		1632	1654		1628		1615	amide I
1608	1609	1609	1607	1608	1608	1609	1609	ν_{8a}
1574	1564	1572	1573	1576	1558	1559	1557	ν_{8b}
	1449			1545	1449	1545	1450	Amide II/II'
	1525		1532		1527		1531	ν_{19a}
1282			1288	1311		1311		amide III
1187	1186	1185	1184	1192	1185	1195	1187	ν_{9a}
868	863	n r	848	868	863	868	863	ν_1

(B) Ternary Complex. Figure 6 compares RR spectra of the TSase ternary complex in H_2O and D_2O, and also upon isotopic substitution of ^{13}C in the carbonyl group (labeled $^{13}C_{17}$) or in the C_{11} methylene group, and of ^2H at the C_{12} and C_{16} ring atoms (labeled 2D). The frequencies are listed in Table 2. $^{13}C_{11}$ substitution does not affect the spectrum; the apparent slight shifts of weak bands are within experi-

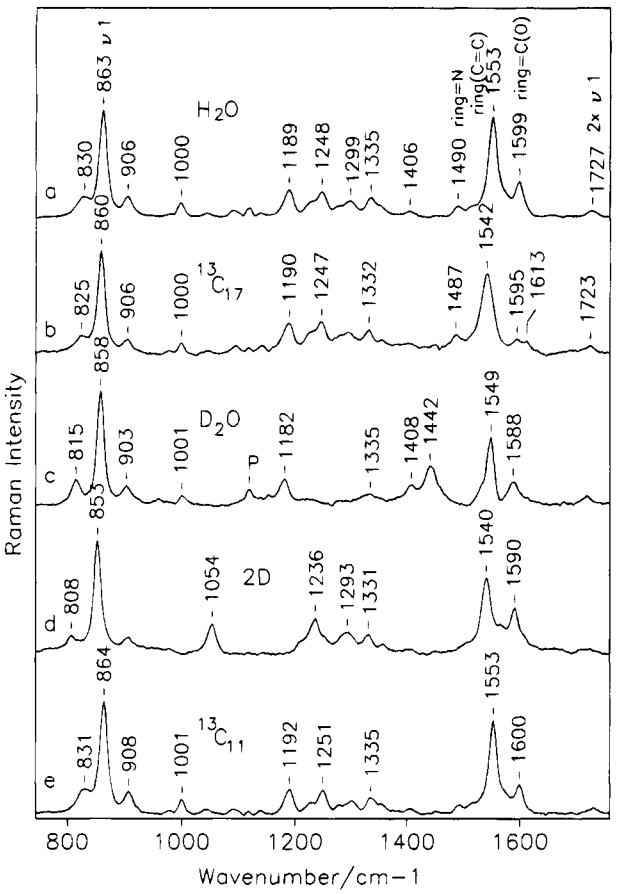

FIGURE 6: 337-nm-excited RR spectra of the TS•FdUMP•CH_2H_4-folate ternary complex (a) in H_2O, (b) with $^{13}C_{17}$ label on the cofactor, (c) in D_2O, where N_{10} and N_{18} hydrogens are exchanged, (d) with D labels at positions 12 and 16 of the PABA-Glu ring of the cofactor, and (e) with ^{13}C at the C_{11} position of the cofactor.

Table 2: Wavenumber Values (cm^{-1}) for 337-nm-excited RR Spectra of TS Ternary Complexes

na	2-D[a]	$^{13}C_{17}$[b]	D_2O[c]	suggested assignments
830	808	825	815	
863	853	860	858	ν_1-like
906	905	906	903	
1000		1000	1001	
1189	1054	1190	1182	ν_{9a}-like
1248	1236	1247		ν_{7a}-like
1299	1293	1295		
1335	1331	1332	1335	
1406			1408	
			1442	amide II′
1490		1487		ring=N
1553	1540	1542	1549	ring (C=C)
1599	1590	(1540)[d]	1588	ring=C(0)

[a] Deuterium substitution at C_{12}-D and C_{16}-D. [b] ^{13}C substitution at C_{17}. [c] Complex formed in D_2O-exchange at N_{10} and N_{18}. [d] Estimated from broadening of 1542-cm^{-1} band.

mental error. The RR band shifts arising from isotope substitutions to the PABA-Glu moiety confirm that the ternary complex RR spectra arise from the PABA-Glu structure, and not from the pteridine ring. The ternary complex of H_4-folate, FdUMP, and TSase, which contains no covalent link between FdUMP and cofactor, gave an RR spectrum identical to the spectrum of the ternary complex with CH_2H_4-folate. This is further evidence that there are no contributions of pteridine vibrations to the observed RR spectra.

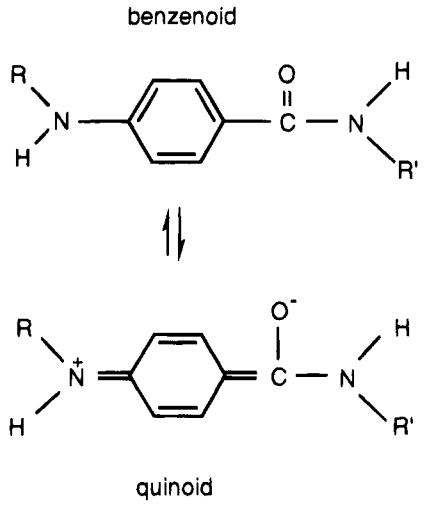

FIGURE 7: PABA-Glu benzenoid and quinoid resonance structures.

The spectral pattern is seen to be very different from that of PABA-Glu and the folates in solution. Instead of a strong invariant ν_{8a} band at 1608 cm^{-1}, one sees a strong band at a much lower frequency, 1553 cm^{-1}, and it is sensitive to D_2O (4 cm^{-1}), and especially to ^{13}C substitution in the carbonyl group (10 cm^{-1}). This band is decidedly not a pure ring mode, in contrast to ν_{8a}. Moreover, there is no ν_{8b} candidate at lower frequency, but rather a moderate intensity band at higher frequency, 1598 cm^{-1}, which is even more sensitive to H/D and carbonyl ^{13}C substitution. There is also a weak lower frequency band, 1490 cm^{-1}, which disappears in D_2O but shifts only slightly on ^{13}C substitution. A search for amide modes reveals an amide II′ candidate in the D_2O solution, at 1442 cm^{-1}, somewhat lower than in PABA-Glu and the folates (1449 cm^{-1}). There is, however, no trace of an amide I band, even though the hydrophobic character of the enzyme binding pocket in the region of the amide bond (Finer-Moore et al., 1990; Matthews et al., 1990) should have produced an amide I frequency well above 1600 cm^{-1} (cf. 1654 cm^{-1} for PABA-Glu in acetonitrile), in a blank region of the spectrum.

To explain these spectral characteristics, we consider the two resonance forms available to PABA-Glu, illustrated in Figure 7, the benzenoid and quinoid forms. For PABA-Glu or the folates in solution, the benzenoid form is clearly dominant, since the RR features are characteristic of substituted benzenes. The very different features seen for the ternary complex can be understood if the protein stabilizes the chromophore largely in the quinoid resonance form. In that case we can assign the dominant 1553-cm^{-1} band to the ring[C=C] stretch; substantial coupling with the ring=C and the ring=N stretches account for the D_2O and ^{13}C sensitivity. The ring=C stretch is assigned to the 1598-cm^{-1} band. Its 10-cm^{-1} D_2O shift results from coupling with the amide N−H bend. The ^{13}C shift should be even larger for this mode, and we suggest that it shifts under the ring[C=C] band at 1542 cm^{-1}, which is noticeably broader than the corresponding bands in the other isotopomers. (There are remnant bands at 1595 and 1613 cm^{-1} in the ^{13}C spectrum, but these are quite weak and may arise from nonfundamental modes.) The ring=N stretch is assigned to the 1490-cm^{-1} D_2O-sensitive band. The absence of amide I is readily understood because the C−O bond order is drastically reduced in the quinoid resonance form. The presence of

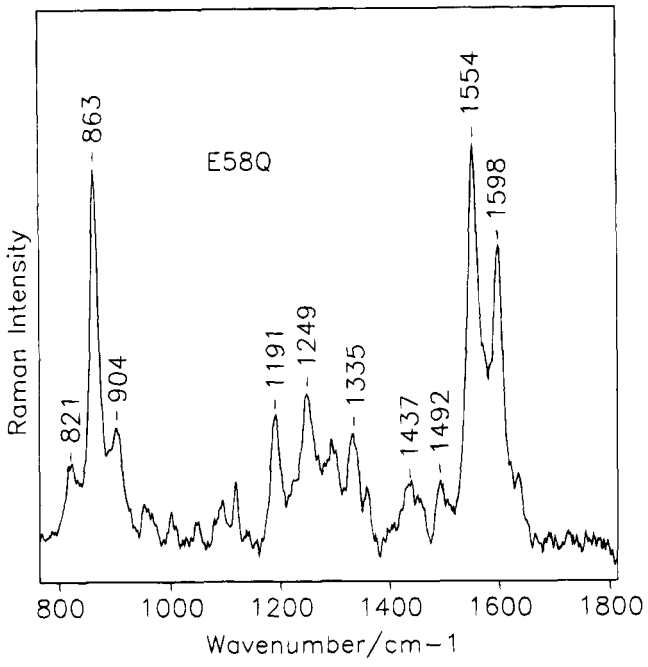

FIGURE 8: 337-nm-excited RR spectrum of the ternary complex of the E58Q mutant of TS with FdUMP and CH_2H_4-folate.

amide II′, at a shifted frequency, is also plausible, since the amide C−N bond order is not greatly affected by whether the adjacent double bond involves the O atom or the ring (Figure 7). Thus, the RR spectrum in the double bond stretching region can readily be interpreted in terms of a shift toward the quinoid resonance form.

At lower frequencies, the bands share some characteristics with benzenoid ring modes. Thus, the strong 863-cm^{-1} band behaves like a standard ν_1 breathing mode, showing a small D_2O sensitivity, similar to that of the folates in solution. The 1189-cm^{-1} band behaves somewhat like the ring mode ν_{9a}, shifting 7 cm^{-1} in D_2O, as in the folates. It shifts 135 cm^{-1} in the 2D spectrum, similar to the ν_{9a} shifts in dideuterated isotopomers of *p*-cresol (Takeuchi et al., 1988). We note, however, that Callender et al. (1988) observed a similar shift, from 1170 to 1060 cm^{-1}, for an RR band of a zinc complex of dimethylaminobenzaldehyde, which was thought to exist in the quinoid resonance form. Thus, a 2D-sensitive band at this frequency does not distinguish between quinoid and benzenoid forms. In general, the assignment of the several bands observed below 1440 cm^{-1} is not at all obvious; their elucidation will require a normal coordinate analysis.

(3) RR Spectra of the E58Q and K48Q Mutants. The carboxylate side chain of Glu58 is close to the PABA N_{10} atom: 4 Å in one structure of the ternary complex (Matthews et al., 1990) and 5 Å in another (Finer-Moore et al., 1990). The distance is long for a direct H-bond, but the carboxylate could serve as a general acid catalyst for the opening of the CH_2H_4-folate imidazoline ring, or else it could stabilize the resulting N_5 imminium ion (Matthews et al., 1990 a,b), in preparation for bond formation with the uracil C_5 atom (Figure 1).

Replacement of Glu58 with asparagine weakens cofactor binding and lowers catalytic activity by several hundred fold (Zapf et al., 1993). Nevertheless, we were able to obtain a RR spectrum of the ternary complex of the E58Q mutant (Figure 8), which is nearly identical with that of the wild type. There are slight differences in relative intensities, and

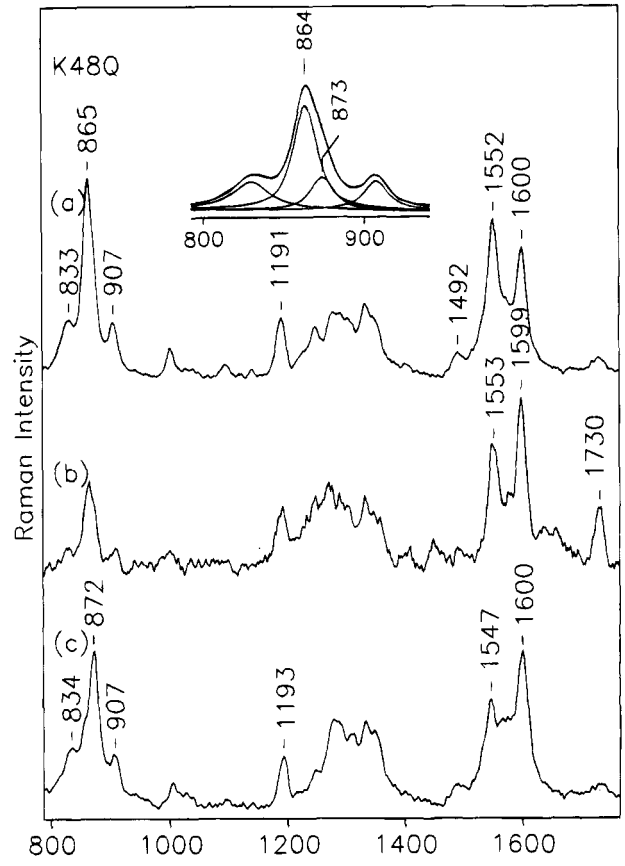

FIGURE 9: RR spectra of the ternary complex of K48Q mutant of TS with FdUMP and CH_2H_4-folate: (a) 337-nm excitation, (b) 356-nm excitation, and (c) the difference spectrum, K48Q minus WT (337-nm spectra), scaled to produce no negative peaks. Inset: expansion of the 865-cm^{-1} region, with band deconvolution.

the weak band at 830 cm^{-1} shifts to 821 cm^{-1} in the mutant. (Thus, the spectrum is not attributable to contamination by native protein.) None of the other frequencies are altered, however, including the bands in the double bond stretching region. The absence of frequency alteration indicates that the Glu58 side chain does not have a strongly polarizing effect on the PABA chromophore (see below).

Another potentially important residue is Lys48, which is favorably placed to interact with the PABA-Glu carboxylate tail of the cofactor (Finer-Moore et al., 1990; Matthews et al., 1990). Accordingly, a mutant in which Lys48 is replaced by glutamine was prepared and examined. The K48Q mutant has greatly reduced activity (5000-fold lower V_{max} with unchanged K_M; unpublished results). The RR spectrum of the K48Q ternary complex is shown in Figure 9 (upper spectrum). It resembles the wild-type spectrum closely, but the 1553- and 1000-cm^{-1} bands are significantly broader, and the 865-cm^{-1} ν_1 band has a distinct shoulder at 872 cm^{-1}, as can be seen in the inset. The spectrum of the wild-type ternary complex can be subtracted from that of K48Q to minimize the 865-cm^{-1} band, leaving a second spectrum (Figure 9, bottom) which is similar but not identical to the wild-type spectrum. In the residual spectrum, ν_1 is higher by 10 cm^{-1} and the ring[C=C] stretch is lower in frequency by 5 cm^{-1}. The ring[C=C] stretch (1547 cm^{-1}) is less intense than the ring=C stretch (1600 cm^{-1}), rather than the reverse. We attribute the residual spectrum to a minority population of cofactor, bound in a different orientation than the majority population; the latter is essentially the same as

in the wild-type structure. In the crystal structure of the K48Q ternary complex, well-defined electron density, similar to that of the wild type, is only observed at one of the two binding sites of the TS dimer (unpublished.) The residual RR spectrum may therefore arise from a minority population of cofactor molecules at the second binding site.

DISCUSSION

The RR spectra establish that the PABA-Glu portion of the cofactor is structurally altered in the TSase ternary complex, relative to PABA-Glu, H_4-folate, or CH_2H_4-folate in solution. The evidence for alteration is particularly clear in the structurally sensitive double bond stretching region, where changes in band frequencies, intensities, and isotope sensitivities all point to a shift in the normal mode compositions. The model compounds in solution all give RR spectral patterns which are recognizably those of a substituted benzene derivative, while the ternary complex does not. The ternary complex spectrum is, however, assignable to the expected modes of a quinoid structure for the PABA-Glu portion of the chromophore. We infer that, while the benzenoid structure is dominant in solution, the quinoid structure is stabilized by the protein. Needless to say, both resonance structures contribute to the overall stretches in both enzyme-bound and solution species, and the relative contributions can only be gauged qualitatively.

This alteration in the electronic structure is expected to red-shift the lowest lying electronic transitions, accounting for the 322- and 370-nm absorption bands of the ternary complex. The longest wavelength absorption bands for CH_2H_4-folate and H_4-folate are 298 and 294 nm in aqueous solution. As a point of comparison, one can cite the 325 → 372-nm shift in the absorption band of dimethylaminobenzaldehyde (in diethyl ether) when it is bound to Zn^{2+}, which shifts the resonance structure toward the quinoid form (Jagodzinsky et al., 1982). Since the same RR bands are enhanced with 337- and 356-nm excitation [the previous report to the contrary (Fitzhugh et al., 1986) being due to an unrecognized photoproduct], both the 322- and 370-nm bands of the ternary complex can confidently be assigned to the PABA-Glu chromophore.

The principal differences between the benzenoid and quinoid structures (Figure 7) are that the quinoid, but not the benzenoid, structure has (1) a dipolar charge distribution and (2) enforced coplanarity of the substituent bonds with the ring, in order to permit sp^2 hybridization at the exocyclic N and C atoms. The protein binding pocket could reinforce one or both of these features. It could provide (1) a complementary charge distribution and (2) steric interactions which favor substituent−ring coplanarity. Indeed, the PABA-Glu substituents are found to be coplanar with the ring in the crystal structures of the TSase ternary complex (Finer-Moore et al., 1990; Matthews et al., 1990). The PABA-Glu geometry is unknown in solution, but it seems likely that amide bonds are displaced from the plane of the ring by steric interactions of the bulky glutamate moiety. The angle between the planes of the amide group and the benzene ring is 26° in the crystal structure of PABA-Glu hydrochloride (Chatterjee et al., 1982), and 17° in the folate analog *N*-[*p*-[[(2,4-diamino-6-quinazyl)methyl]amino]benzoyl]diethylaspartate dihydrate (Mastropaolo et al., 1986). It may be significant in this connection that TSase undergoes an extensive conformational change upon cofactor binding (Stroud & Finer-Moore, 1993). Perhaps this conformation change enforces the PABA-Glu planarity, and thereby converts part of the binding energy into stabilization of the quinoid structure.

With respect to charge effects, the only polar residues in the vicinity of the PABA-Glu are Glu58 and Lys48. These residues are in fact complementary to the PABA-Glu quinoid form, since the Glu48 carboxylate group is near the positively charged amine N_{10}, and the Lys48 side chain is not far from the negatively charged amide O_{17} atom. While the distances are too large to permit H-bonding, the electrostatic fields are nevertheless expected to stabilize the quinoid charge distribution, although these fields might be attenuated by water molecules, a number of which are found in the active site (Fauman et al., 1994). It is surprising that replacement neither of Glu58 nor of Lys48 by glutamine produced a change in the RR spectrum of the ternary complex in the direction of a benzenoid pattern. The E58Q mutant produced some alteration in relative intensities and a small shift in an unidentified 830-cm^{-1} band, but no change in the frequencies of the bands in the double bond region. The K48Q mutant gave RR evidence for two cofactor populations, the majority having a spectrum identical to the wild type. The minority RR spectrum showed some frequency and intensity changes, but not a significant shift toward the benzenoid pattern. We conclude that neither Glu58 nor Lys48 is essential to maintenance of the quinoid structure, although they may well add to its stabilization. It appears that steric enforcement of planarity by the protein is sufficient to shift the PABA-Glu structure from benzenoid to quinoid.

Glu58 and Lys48 may nevertheless aid in catalysis, and indeed their replacement does lower enzyme activity substantially. As noted above, Glu58 is strategically placed to deliver a proton to N_{10} as part of the ring-opening process. The ring opening itself may be induced by the enforced planarity of the PABA-Glu via the resulting strain in the methylene bridge between N_{10} and N_5 of the CH_2H_4 folate cofactor (Figure 1). Thus, Glu58 may act in concert with the steric forces that stabilize the quinoid structure. These forces are, however, sufficient in themselves to induce ring opening, the critical first step in the enzyme mechanism, as evidenced by the fact that the E58Q mutant does turn over, albeit at a reduced rate.

In summary, we infer from the RR evidence that the PABA-Glu portion of the TSase cofactor adopts the quinoid form in the ternary complex, due to steric forces that enforce amide−ring coplanarity. These forces, which are no doubt generated by the protein conformation change that accompanies cofactor binding, activate the cofactor by inducing the methylene bridge to open. The Glu58 and Lys48, whose replacement does not significantly alter the RR spectrum, must be involved in catalytic steps that are subsequent to cofactor activation.

ACKNOWLEDGMENT

We thank Robert Pascal and Doug Ho for helpful discussions and Bruce Dunlap for kindly providing the dihydrofolate reductase used in preparing the isotopic cofactor. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

REFERENCES

Austin, J. C., Kuliopulos, A., Mildvan, A. S., & Spiro, T. G. (1992) *Protein Sci. 1*, 269.

Bacquet, R. J., Bartlett, C. A., Morse, C. A., Booth, C. L. J., Herrmann, S. M., Howland, E. F., Ward, R. W., & White, J. (1992) *J. Med. Chem. 35*, 663.

Bajorath, J., Kitson, D. H., Fitzgerald, G., Andzelm, J., Kraut, J., & Hagler, A. T. (1991) *Proteins 9*, 217.

Callender, R. H., Chen, D., Lugtenberg, J., Martin, C., Rhee, K. W., Sloan, D., Vandersteen, R., & Yue, K. T. (1988) *Biochemistry 27*, 3672.

Chatterjee, C., Dattagupta, J. K., & Saha, N. N. (1982) *Acta Crystallogr., Sect. B 38*, 2086.

Cisneros, R. J., Silks, L. A., & Dunlap, R. B. (1988) in *Drugs of the Future,* Silks, L. A., (Ed.) pp 859−881, Wiley, New York.

Cutmore, E. A., & Hallam, H. E. (1969) *Spectrochim. Acta 25A*, 1767.

Danenberg, P. V., Langenbach, R. J., & Heidelberger, C. (1974) *Biochemistry 13*, 926.

Dollish, F. R., Fateley, W. G., & Bentley, F. F. (1974) *Characteristic Raman Frequencies of Organic Compounds*, Wiley & Sons, New York.

Donato, H., Jr., Aull, J. L., Lyon, J. A., Reinsch, J. W., & Dunlap, R. B. (1976) *J. Biol. Chem. 231*, 1303.

Fauman, E. B., Rutenber, E. E., Maley, G. F., Maley, F., & Stroud, R. M. (1994) *Biochemistry 33*, 1502.

Finer-Moore, J. S., Montfort, W. R., & Stroud, R. M. (1990) *Biochemistry 29*, 6977.

Fitzhugh, A. L., Fodor, S., Kaufman, S., & Spiro, T. G. (1986) *J. Am. Chem. Soc. 108*, 7422.

Friedkin, M. (1959) *Fed. Proc. 18*, 230.

Hachey, D. L., Pallidino, L., Blair, J. A., Rosenberg, I. H., & Klein, P. D. (1978) *J. Labelled Compd. Radiopharm. 14*, 479−486.

Jagodzinski, P. W., Funk, G. F., & Peticolas, W. L. (1982) *Biochemistry 21*, 2193.

James, T. L., Pogolotti, A. L., Jr., Ivanetich, K. M., Wataya, Y., Lam, S. M., & Santi, D. V. (1976) *Biochem. Biophys. Res. Commun. 72*, 404.

Kniseley, R. N., Fassel, V. A., Farquhar, E. L., & Gray, L. S. (1962) *Spectrochim. Acta. 18*, 1217.

Mastrapaolo, D., Smith, H. W., Camerman, A., & Camerman, N. (1986) *J. Med. Chem. 29*, 155.

Matthews, D. A., Villafranca, J. E., Janson, C. A., Smith, W. W., Welsh, K., & Freer, S. (1990) *J. Mol. Biol. 214*, 937.

McNamara, D. J., Berman, E. M., Fry, D. W., & Werbel, L. M. (1990) *J. Med. Chem. 33*, 2045.

Osborn, M. J., Talbert, P. T., & Muennekens, F. M. (1960) *J. Am. Chem. Soc. 82*, 4921.

Plante, L. T., Williamson, K. L., & Pastore, E. J. (1980) *Methods Enzymol. 65*, 533−535.

Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins: Vol. 1, Chemistry and Biochemistry of Folates* (Blakely, R. L., & Benkovic, S. J., Eds.) pp 345−399, John Wiley & Sons, New York.

Santi, D. V., McHenry, C. S., & Sommer, H. (1974) *Biochemistry 13*, 471.

Takeuchi, H., Watanabe, N., & Harada, I. (1988) *Spectrochim. Acta 44A*, 749.

Varney, M. D., Marzoni, G. P., Palmer, C. L., Deal, J. G., Webber, S., & Welsh, K. M. (1992) *J. Med. Chem. 35*, 663.

Zapf, J. W., Weir, M. S., Emerick, V., Villafranca, J. E., & Dunlap, R. B. (1993) *Biochemistry 32*, 9274.

BI942301P