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Trimethoprim Binding to *Lactobacillus casei* Dihydrofolate Reductase: A ^{13}C NMR Study Using Selectively ^{13}C-Enriched Trimethoprim†

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ABSTRACT: We have measured the ^{13}C chemical shifts for trimethoprim molecules selectively enriched with ^{13}C at the 2-, 4-, 5-, 6-, and 7-positions and the *p*-OCH_3 position in their complexes with *Lactobacillus casei* dihydrofolate reductase in the presence and absence of coenzyme analogues. The C2 carbon shifts indicate that the pyrimidine ring is protonated at N1 in all the complexes of trimethoprim with the enzyme and coenzymes and in each case the pyrimidine ring is binding in a similar way to that of the corresponding part of methotrexate in the enzyme–methotrexate complex. The C6 carbon of trimethoprim shows a large upfield shift in all complexes (3.51 to 4.70 ppm) but no shift in the complex of 2,4-diaminopyrimidine with the enzyme: these shifts probably arise from steric interactions between the C1′ and C2′ carbons and the H6 proton, which approach van der Waals contact in the folded conformation adopted by trimethoprim when bound to the enzyme. The large shift observed for C6 in all complexes indicates that the basic folded conformation is present in all of them. A comparison of the ^{13}C shifts in the enzyme–trimethoprim–NADPH complex with those in the enzyme–trimethoprim binary complex shows substantial changes even for carbons such as C6 and *p*-OCH_3 (0.46 and −0.36 ppm, respectively), which are remote from the coenzyme: these are caused by ligand-induced conformational changes that may involve displacement of the helix containing residues 42–49. In the ternary complex with $NADP^+$, the two conformational states previously described are further characterized: separate signals are seen for conformations I and II for the C2, C4, C5, C6, and C7 carbons. One set of chemical shifts has values similar to those measured in the binary complex with trimethoprim and also to those in the ternary complex with the methyl β-riboside of 2′-phosphoadenosine 5′-(diphosphoribose); these are assigned to conformation II of the complex. The complex of [*m-methoxy*-^{13}C]brodimoprim [[*3′-methoxy*-^{13}C]- or [*5′-methoxy*-^{13}C]-2,4-diamino-5-(3′,5′-dimethoxy-4′-bromobenzyl)pyrimidine] with the enzyme shows two equal-intensity ^{13}C signals at 274 K, which coalesce to a single absorption when the temperature is raised to 287 K. This two-site exchange between nuclei at the C3′ and C5′ positions has been characterized in terms of "ring flipping", and the rate of this process can be estimated to be 65 ± 8 s^{-1} at 287 K. For the ternary complex with $NADP^+$, the ^{13}C spectrum showed the same coalescence temperature (287 K) as observed for the binary complex: at this temperature the ternary complex is predominantly in form II (75%).

The antibacterial drug trimethoprim (TMP)[1] acts by selectively inhibiting the enzyme dihydrofolate reductase in bacterial cells. Considerable efforts have been made to understand the factors controlling the specificity of trimethoprim binding to the bacterial enzyme, and numerous structure–activity studies using trimethoprim-related inhibitors have been reported (Roth & Cheng 1982). Previously, we have used NMR methods to investigate the protonation state of the pyrimidine ring of bound trimethoprim (Roberts et al., 1981; Bevan et al., 1985), to determine its conformation in complexes with both bacterial and mammalian dihydrofolate reductase (Cayley et al., 1979; Birdsall et al., 1983), and to investigate the presence of multiple conformations in these complexes (Gronenborn et al., 1981a,b; Birdsall et al., 1984). One of the major problems encountered in studying complex NMR spectra is that of assigning the signals to particular nuclei in the protein–ligand complexes. This can be largely overcome by using isotopic labeling to simplify the NMR spectra of the proteins or their bound ligands. Isotopic labeling with ^{13}C

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[1] Abbreviations: TMP, trimethoprim; DAP, 2,4-diaminopyrimidine; PADPR-OMe, methyl β-riboside of 2′-phosphoadenosine 5′-(diphosphoribose); NIC, nicotinamide; DMF, dimethylformamide.

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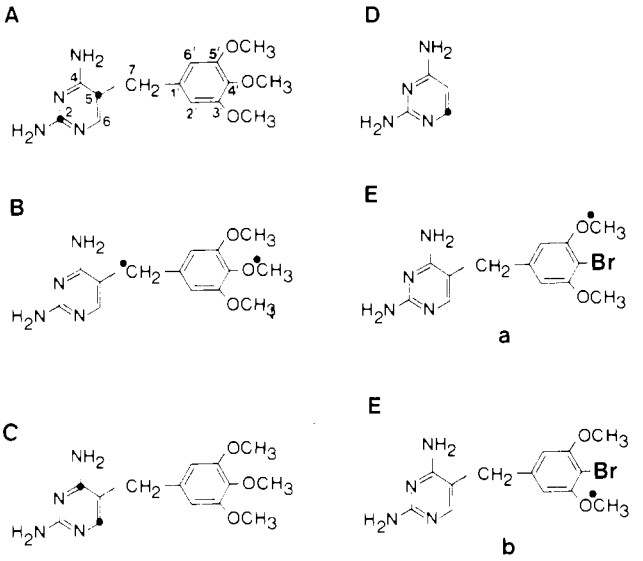
Chart I[a]



[a] A, [2,5-^{13}C]trimethoprim; B, [7,*4'-methoxy*-^{13}C]trimethoprim; C, [4,6-^{13}C]trimethoprim; D, [6-^{13}C]diaminopyrimidine; E, [*m-methoxy*-^{13}C]brodimoprim.

(natural abundance 1.1%) at selective sites is particularly advantageous because it leads immediately to the assignment of the resonances and also to a dramatic improvement in the sensitivity of the experiment. Studies of the chemical shifts and relaxation times of assigned ^{13}C nuclei in proteins and protein complexes can provide useful information about ionization states, changes in conformation, the presence of multiple conformational states, and dynamic processes in the system. We have been using this approach to provide such information about complexes of trimethoprim and some of its analogues with the enzyme dihydrofolate reductase. The ^{13}C-labeled trimethoprim molecules synthesized for this purpose are listed in Chart I.

The compounds having ^{13}C labels in or near the pyrimidine ring (A–C) can be used to monitor the protonation state of the pyrimidine ring in complexes of trimethoprim with the enzyme and coenzyme analogues. In addition, the ^{13}C chemical shifts of all the labels should be sensitive to any conformational changes that accompany complex formation.

The ternary complex formed by trimethoprim and NADP$^+$ with the *Lactobacillus casei* enzyme is already known to exist in two conformational forms present in almost equal amounts; the extensive ^{13}C labeling in compounds A–E allows us to explore the influence of the different conformational states on the environments of the ^{13}C nuclei of trimethoprim. The ^{13}C labels should also provide a sensitive method of detecting multiple conformational states, if present, in ternary complexes with other coenzyme analogues.

In the complex of trimethoprim with the enzyme, one can envisage several dynamic processes involving the trimethoxybenzyl ring. Two of these, ring flipping and conformational interconversion of the out-of-plane 4'-methoxy group, could be correlated motions: such processes can be investigated by examining the temperature dependence of the ^{13}C signals from the labeled methoxy groups in compounds B and E in their complexes with the enzyme.

Materials and Methods

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as described previously (Dann et al., 1976). The synthesis of ^{13}C-labeled analogues of trimethoprim (compounds A–C) and of [6-^{13}C]-2,4-diaminopyrimidine (D) (DAP) is being described elsewhere (Cheung et al., 1985). [*m-methoxy*-^{13}C]Brodimoprim [[*3'-methoxy*-^{13}C]- or [*5'-methoxy*-^{13}C]-2,4-diamino-5-(3',5'-dimethoxy-4'-bromobenzyl)pyrimidine] (E) was prepared from 2,4-diamino-5-(3'-hydroxy-4'-bromo-5'-methoxybenzyl)pyrimidine by methylation with ethyl [^{13}C]bromomethanoate in the presence of K_2CO_3 in DMF as described by Kompis and Then (1984).

The samples used in the NMR studies contained 0.35–1.0 mM enzyme, 50 mM potassium phosphate, and 500 mM potassium chloride in 1.3–3 mL of ^2H_2O (pH* 6.5). The trimethoprim analogues were added in equimolar amounts to the enzyme as aliquots dissolved in microliter volumes of [$^2H_6$]Me_2SO.

The ^{13}C NMR spectra of the complexes of the trimethoprim analogues (A–D) were obtained at 67.9 MHz on a Bruker WH270 spectrometer. Some of these spectra were acquired under conditions of ^{1}H broad-band decoupling with a composite-pulse low-power decoupler (Freeman et al., 1983). Spectra were usually obtained at 298 K (some measurements were at 277–313 K) with a spectral width of 16 kHz and 16384 data points (final digital resolution 1.95 Hz/point): the pulse interval was 0.5 s, and a flip angle of 30° was usually used. A total of 50000 or more transients were averaged, and before Fourier transformation the free induction decay was multiplied by an exponential function to improve the signal-to-noise ratio leading to additional line broadening of 5–30 Hz. Chemical shifts were measured in ppm from external dioxane (1:1 dioxane/^2H_2O), with upfield shifts positive.

The ^{13}C spectra of the complexes with labeled brodimoprim (E) were obtained over the temperature range 278–308 K on a Bruker WM200 spectrometer operating at 50.2 MHz. A DEPT pulse sequence

$$^1H \quad \left(\frac{\pi}{2}\right)_{(x)} - D_2 - \pi_{(x)} - D_2 - \theta_y - D_2$$

$$^{13}C \quad \left(\frac{\pi}{2}\right)_{(x)} - \pi_{(x)} - \text{FID}$$

where $D_2 = 1/(2J)$ and $\theta_y = 0.2\pi$ (Doddrell et al., 1982), was used to enhance the signal-to-noise by polarization transfer prior to detecting the free induction decay (FID). Proton decoupling was achieved by selective irradiation at the ^{1}H frequency with high power during the acquisition period and low power during a pulse delay of 2 s. This procedure prevented overheating of the high ionic strength solutions.

Results and Discussion

The use of ^{13}C selectively enriched trimethoprim in these studies allows us to obtain excellent ^{13}C NMR spectra from samples containing about 1.5 mL of 0.5 mM enzyme in the presence of equimolar amounts of trimethoprim in 12 h (see Figure 1). Although three of the compounds contain more than one labeled site, there was no ambiguity in the assignments since the carbons in compounds B and C can be characterized by the number of directly bonded protons while those in compound A can be assigned by comparison with the ^{13}C spectrum of the 2-^{13}C-labeled compound examined previously (Roberts et al., 1981). Table I summarizes the ^{13}C chemical shifts of trimethoprim in the various complexes measured with respect to the shifts of the corresponding carbons in protonated free trimethoprim.

Previous studies using ^{13}C- and ^{15}N-labeled trimethoprim (Roberts et al., 1981; Cocco et al., 1983; Bevan et al., 1985) have provided unequivocal evidence that the N1 position of the pyrimidine ring in trimethoprim is protonated in its binary complex with the enzyme. In complexes formed with [2,5-

Table I: ^{13}C Chemical Shifts[a] of Trimethoprim in Its Complexes with *L. casei* Dihydrofolate Reductase at 298 K

carbon	analogue	free protonated[b] TMP[c]	TMP–enzyme	TMP–enzyme–PADPR-OMe	TMP–enzyme–NADP$^+$ form I	TMP–enzyme–NADP$^+$ form II	TMP–enzyme–NADPH
2-C	A	−87.60	−1.28	−1.22	−1.50	−1.21	−1.09
5-C	A	−42.37	−1.21	−1.46	0.25	−1.31	−1.08
p-OCH_3	B	5.92	−0.07	−0.29	−0.25[d]	−0.07[d]	−0.43
7-CH_2	B	34.34	0.36	0.31	−0.48	0.45	−0.18
6-CH	C	−73.11	4.24	4.35	3.51	4.29	4.70
4-C	C	−97.86	−0.47	−0.70	−0.33	−0.80	−0.30

[a] Chemical shifts measured in ppm (±0.05 ppm) from corresponding carbon shift in free protonated TMP. [b] pH 2. [c] Chemical shifts measured in ppm (±0.05 ppm) from dioxane external reference (dioxane/^2H_2O, 1/1). [d] Signals not resolved at 298 K; chemical shifts of the two forms estimated from the observed line separation at 277 K.

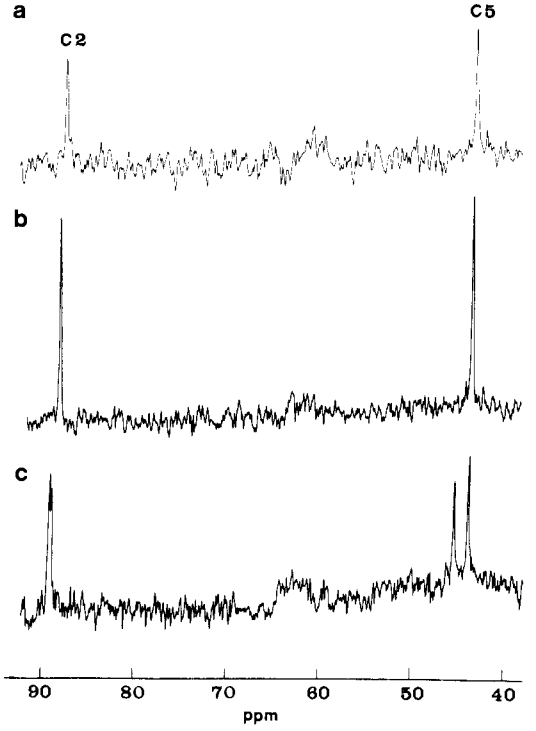


FIGURE 1: 67.91-MHz ^{13}C NMR spectrum at 298 K of [2,5-^{13}C]-trimethoprim in the presence of (a) 1 equiv of *L. casei* dihydrofolate reductase, (b) 1 equiv of dihydrofolate reductase and 1 equiv of NADPH, and (c) 1 equiv of dihydrofolate reductase and 1 equiv of NADP$^+$. Chemical shifts are in ppm downfield from dioxane external references.

^{13}C]trimethoprim (A), the 2-^{13}C chemical shift in the bound state is found to be fairly close to that of protonated trimethoprim in the absence of enzyme (1.29 ppm downfield from the signal in the protonated form and 5.8 ppm upfield of that in the nonprotonated form).

This is also true in the ternary complexes of enzyme, trimethoprim, and NADP$^+$, NADPH, or PADPR-OMe. It is clear that in each case the trimethoprim is binding with its pyrimidine ring protonated at the N1 position as previously noted in our ^{15}N studies (Bevan et al., 1985). Thus, if we compare the ^{13}C chemical shifts observed for other carbons in free protonated trimethoprim with those of the corresponding carbons in the various complexes (see Table I), we can estimate the total shielding contributions arising from interactions with the protein and from any conformational changes induced in the trimethoprim structure on binding. The extra bound shift (−1.28 ppm) is very similar to that observed for the corresponding carbon in methotrexate in its complex with the enzyme (−1.32 ppm) (Cocco et al., 1981a,b, 1983), indicating that the pyrimidine ring of trimethoprim is binding in the same site as the corresponding part of the pteridine ring of methotrexate. Large upfield shifts are observed for the pyrimidine C6 carbon in all the complexes studied (4.70 to 3.51 ppm) except for those containing [6-^{13}C]-2,4-diaminopyrimidine (D), where the C6 is essentially unshifted: these shifts appear to be caused by direct interactions at C6 from the benzyl ring.

Binary Complex of the Enzyme with Trimethoprim. The conformation of trimethoprim in its complex with *L. casei* dihydrofolate reductase has been determined previously (Cayley et al., 1979; Birdsall et al., 1983) by a combination of NMR data (ring current shift calculations and NOE measurements) and X-ray data obtained from related complexes (Matthews et al., 1978). Trimethoprim was shown to adopt a folded conformation in the bound state with one of the benzyl aromatic protons (H2′ or H6′) near to the pyrimidine H6 proton. The crystal structure of the *E. coli* enzyme–trimethoprim complex (Baker et al., 1981; Matthews et al., 1985) indicates that a similar conformation exists in the crystalline state. The N1 atom of trimethoprim is close to the conserved Asp-26 residue [as is N1 of methotrexate in the enzyme–methotrexate–NADPH crystal structure (Matthews et al., 1978; Bolin et al., 1982)]. It seems certain that the protonated N1 interacts electrostatically with the anionic carboxylate group of Asp-26. Evidence that the proton on N1 also forms a hydrogen bond to one of the carboxylate oxygen atoms has been obtained from ^1H NMR studies on enzyme complexes with ^{15}N-labeled trimethoprim (Bevan et al., 1985). By building trimethoprim into the crystal structure of the *L. casei* enzyme (Bolin et al., 1982), we can identify the residues that are closest to the trimethoprim atoms, and Table II summarizes the relevant internuclear distances between atoms in trimethoprim and in the enzyme and coenzyme.

We have already mentioned that in the binary trimethoprim–*L. casei* enzyme complex, the C6 carbon of the pyrimidine ring shows a very large upfield shift (4.24 ppm). Calculations of ring current shift contributions (Johnson & Bovey, 1958) from nearby aromatic residues (such as Phe-30 and Phe-49) and from the trimethoprim benzyl ring indicate fairly small contributions at the C6 carbon (<0.1 ppm): thus, the large observed shift at C6 cannot be explained in terms of ring current shifts. Because of the proximity of the C6 position to the charged Asp-26 carboxylate group, we must also consider the possibility that the shift arises from an electric field effect (Feeney et al., 1966; Batchelor et al., 1975). If such shielding effects are large, then they should also be present in complexes of the enzyme with 2,4-diaminopyrimidine (DAP) (D), which is known to bind in a similar manner to the corresponding part of trimethoprim (Birdsall et al., 1977, 1983; Cayley et al., 1979). However, there was no change in the shift of the C6 carbon of DAP when [6-^{13}C]DAP was examined at low concentration (0.20 mM) in the presence of enzyme (0.62 mM), where 40% of the DAP is known to be bound. This suggests that the large shift observed for C6 in bound trimethoprim is not related to electric

Table II: Atoms in the Enzyme and Coenzyme in Close Proximity to Trimethoprim Atoms in the Ternary Complex *L. casei* Enzyme–TMP–NADPH

trimethoprim atom	nearby atoms and distances (Å)									
C2	Leu-4		Trp-5		Ala-6		Asp-26		Phe-30	
	Hγ	3.3	Hα	3.3	N	3.8	$O\delta_1$	3.6	$H\beta_1$	3.5
	$H\delta_1$	>3.6	Cα	4.0	Cβ	4.0	$O\delta_2$	3.8	$C\delta_2$	4.0
			C=O	3.7	Hβ	3.0-4.8			H6	3.0
C4	NIC		Leu-4		Trp-5		Phe-30			
	O	3.4	H6	3.1	Hα	2.8	$C\epsilon_2$	3.6		
	C4	4.0					H5	3.2		
	H4	3.1					$C\delta_2$	3.4		
							H6	2.8		
C5	NIC		Trp-5		Phe-30					
	O	3.4	Hα	3.9	$C\epsilon_2$	3.7				
	C4	3.9			H5	3.6				
	H4	3.4			$C\delta_2$	3.5				
					H6	3.1				
C6	NIC		Ala-6		Asp-26		Phe-30			
	O	3.8	Hβ	3.6–5.3	$O\delta_1$	3.6	$C\delta_2$	3.9		
							H6	3.5		
C7	NIC		Phe-30							
	H4	3.0	H5	3.7						
3-OCH_3	NIC		Leu-19[a]		His-18					
	O2′	2.6	Hγ	1.4	Hα	3.7				
	C2′	3.7	Cγ	2.1	C	3.6				
			$C\delta_1$	3.3						
			$C\delta_2$	2.2						
			$H\delta_1$	3.2–4.0						
			$H\delta_2$	1.6–3.1						
4-OCH_3	Ser-48		Phe-49		Pro-50		Leu-19			
	O	3.5	Hα	3.6	$H\delta_2$	3.7	$H\delta_1$	3.8–5.2		
5-OCH_3	Leu-27		Phe-30							
	$C\delta_2$	3.3	H2	3.8						
	$H\delta_2$	2.4–4.0								

[a] Several of these Leu-19 atoms are estimated to be nearer to the TMP than is allowed by the van der Waals radii; in this case, the side chain of Leu-19 can take up other conformations with more favorable van der Waals contacts.

field effects or other interactions involving Asp-26 since these would have similarly influenced the C6 of bound DAP. The absence of a bound shift for C6 in DAP also indicates that there is no appreciable "solvent" shift accompanying the transfer of DAP from aqueous solution to its environment in the protein complex. A consideration of the bound conformation of trimethoprim strongly suggests that the large observed shift at the C6 position in bound trimethoprim (4.24 ppm) results from steric interactions between the 6-proton and the nearby C1′ and C2′ atoms of the benzyl ring. In the bound conformation of trimethoprim as determined from our previous NMR studies (Birdsall et al., 1983) ($\theta_1 = 193 \pm 10°$ and $\theta_2 = 73 \pm 10°$), these benzyl carbon atoms are 2.55 and 2.4 Å, respectively, from the pyrimidine C6 proton. It is difficult to quantitate the ^{13}C chemical shift contributions from such steric interactions, but they are known to be capable of producing upfield shifts of more than 5 ppm (Stothers, 1972; Levy et al., 1980; Grant & Cheney, 1967). The H2′ proton is also near to H6 (2.4 Å) but not sufficiently close to cause steric compression. This large chemical shift at C6 provides a useful qualitative indication of any changes in trimethoprim conformation in ternary complexes with coenzyme analogues.

The significant downfield shift observed for C2 (−1.28 ppm) has not, as yet, been explained. One possibility is that it arises from anisotropic shielding effects of the Asp-26 carboxylate group, which also hydrogen bonds to one of the 2-NH_2 protons. It is also possible that the deshielding arises from changes in charge density on C2, which accompany the interactions of Asp-26 carboxylate with the protonated N1 of TMP.

Significant chemical shift changes are also observed for the other atoms in trimethoprim in its binary complex with the enzyme (C4, −0.47 ppm; C5, −1.21 ppm; C7, 0.36 ppm; *p*-OCH_3, −0.07 ppm). Calculations of ring current chemical shifts (Johnson & Bovey, 1958) indicate that significant contributions from this source can only be expected at the C4 (0.38 ppm), C5 (0.29 ppm), and C7 (−0.14 ppm) positions: for C4 and C5, the contributions are mainly from Phe-30 and the trimethoprim benzyl ring while for C7 the contributions are from Phe-30 and Phe-49. In each case, the ring current shift contribution is opposite in sign to the observed shift, indicating that there must be a larger shielding contribution from some other source.

Ternary Complexes of Enzyme with Trimethoprim and Coenzyme Analogues. From Tables I and II, it is clear that some carbons in trimethoprim that cannot be in direct contact with coenzyme (such as C6 and *p*-OCH_3) show substantial changes in ^{13}C shift in the ternary complex when compared with the values in the binary complex. These must be due to ligand-induced conformational effects. In an attempt to understand the origin of these effects, we have examined complexes formed not only with the oxidized and reduced coenzyme but also with PADPR-OMe—a coenzyme analogue in which the nicotinamide ring has been replaced by a methoxy group. This should allow us to separate out conformational effects arising from the binding of the adenine ring (whose site is remote from the trimethoprim) from the direct and conformational effects due to nicotinamide ring binding. If the enzyme–trimethoprim–PADPR-OMe complex has a similar structure to that of the enzyme–trimethoprim–NADPH complex, none of the trimethoprim atoms are expected to be close to the coenzyme atoms. The largest shifts in this ternary complex relative to those in the binary one are seen at the C4 (−0.23 ppm), C5 (−0.25 ppm), and *p*-OCH_3 (−0.22 ppm) carbons.

Matthews et al. (1979) have pointed out that several residues on the helix 42–49 are involved in coenzyme binding: Arg-43

(to 2′-PO_4 group), Arg-44 (to pyrophosphate), Ser-48 (to nicotinamide ribose O2′), and Thr-45 (to adenine ribose O2′). This helix might undergo some movement when the coenzyme binds. We have recently obtained evidence for this by observing ^1H chemical shift changes on coenzyme binding for residues such as Tyr-46 and Phe-49 (which are in the helix) and Val-61 and Tyr-68 (which are in contact with residues in the helix) (S. J. Hammond, B. Birdsall, J. Feeney, G. C. K. Roberts, and M. S. Searle, unpublished results). Previously, we have reported cooperativity in the binding of trimethoprim with coenzyme analogues (~5-fold increase in TMP binding in the presence of PADPR-OMe), which would also be consistent with such conformational changes (Birdsall et al., 1980). Examination of Table II indicates that several protein residues near to trimethoprim could be influenced by movements of helix 42–49. For example, Ser-48 and Phe-49 at the end of the helix are close to the para position of the benzyl ring and could contribute to the shielding of the p-OCH_3 carbon. The small change in shielding (0.1 ppm) of the C6 carbon on forming the ternary complex with PADPR-OMe indicates that the overall conformation of the trimethoprim itself is not substantially different from that in the binary complex.

When we examine the ternary complex with NADPH, the C6 carbon again shows a large shift compared with that in protonated free trimethoprim, indicating that the folded structure is still present. However, it does have a somewhat different shift (4.70 ppm) from the binary complex (4.24 ppm) and ternary complex with PADPR-OMe (4.35 ppm), which indicates some change in the orientation of the benzyl ring with respect to the pyrimidine ring. The carbon at C7, which had a similar shift in the binary complex and in the ternary complex with PADPR-OMe, has experienced a downfield shift of 0.53 ppm. The reduced nicotinamide ring is expected to make direct contact with the C7 protons, so this change is not surprising, although the detailed origin of the deshielding is obscure. The shielding of the p-OCH_3 carbon is again decreased (compared with binary complex) to a similar extent as observed in the PADPR-OMe ternary complex, presumably by conformational changes related to movements of the helix 42–49.

The complex of trimethoprim and $NADP^+$ with *L. casei* dihydrofolate reductase has been shown to exist as a mixture of approximately equal amounts of two slowly interconverting conformations in solution, and these have been characterized previously by NMR (Gronenborn et al., 1981a,b; Birdsall et al., 1984). Characteristic ^1H and ^{31}P chemical shifts of nuclei in the coenzyme in the two conformations formed with different trimethoprim and coenzyme analogues indicate that the nature of the two conformations is the same in the different complexes. The major difference identified so far is that the nicotinamide ring binds differently in the two conformations. In form I the nicotinamide ring is closely bound to the enzyme while in form II it appears to be away from the enzyme and extending into solution. There are also differences in the nicotinamide ribose and in the conformation of the pyrophosphate moiety in the two forms. Previous studies have shown that for PADPR-OMe (which does not have a nicotinamide ring) the conformation of the ternary complex with trimethoprim is very similar to that in form II (Hyde, 1981).

The presence of the two conformational states in the enzyme–$NADP^+$–trimethoprim complex is confirmed in the present studies by the fact that two ^{13}C signals are observed for each of the labeled carbons at C2, C4, C5, C6, and C7 positions (see Table I). Form II is expected to give shifts that are similar to those in the binary complex and also in the ternary complex with PADPR-OMe: Consideration of the data in Table I reveals that one of the two signals observed for each carbon is usually close to the value in the binary complex. A large shift difference between the two forms is seen at the C7 position (−0.93 ppm), which no doubt reflects the fact that C7 is the trimethoprim carbon that is closest to the nicotinamide ring in conformation I. There is also a substantial shift difference at C4, and this is also near to the nicotinamide ring in form I. A shift difference (−0.78 ppm) is also seen at C6, indicating that there could be some difference in orientation of the benzyl ring relative to the pyrimidine ring in the two forms of the complex: however, a large steric shift (4.29 and 3.51 ppm) is observed in each case, showing that the basic folded conformation is maintained in each form. Previous studies on the ^1H chemical shifts of bound trimethoprim in the ternary complex had indicated that this was the case for form I but were unable to provide similar information for form II (Birdsall et al., 1984).

Study of Dynamic Processes in Bound Trimethoprim. In our previous ^1H NMR studies in binary trimethoprim complexes with the enzyme, we obtained indirect evidence that a dynamic process was influencing the signals from the 2′,6′-protons of the benzyl ring (Cayley et al., 1979). Saturation-transfer methods were used to locate the frequencies of signals from bound trimethoprim by observing the intensities of the signals in free trimethoprim as a function of a selective irradiation frequency. In these experiments, only a single frequency (over a fairly broad range ~130 Hz) could be detected for the bound H2′ and H6′ protons, indicating that they were each in the same or an averaged environment. Difference spectroscopy between spectra obtained for the *L. casei* enzyme–trimethoprim complex formed with selectively deuterated trimethoprim ([2′,6′-^2H]TMP) and with unlabeled trimethoprim confirmed that the bound H2′,H6′ signal was very broad even at 318 K (Cayley et al., 1979). (A similar experiment using the *Escherichia coli* enzyme gave a single absorption for the H2′ and H6′ protons in its spectrum at 318 K.) We postulated that flipping of the benzyl ring about its C1′–C4′ symmetry axis in the *L. casei* enzyme complex was incompletely averaging the environments of the H2′ and H6′ protons and resulting in the single broad absorption band. The ^1H chemical shift difference between the H2′ and H6′ protons was estimated to be 320 Hz at 270 MHz (Cayley et al., 1979), and at coalescence the rate can be estimated to be $>1500\ s^{-1}$.

From neutron diffraction crystal studies of free trimethoprim (Koetzle & Williams, 1976) and X-ray studies on trimethoprim bound to *E. coli* dihydrofolate reductase (Baker et al., 1981; Matthews et al., 1985) the p-OCH_3 group is known to be out of the plane of the benzyl aromatic ring. Since steric interactions with the neighboring methoxy groups are the cause of the nonplanarity, it seems likely that this methoxy group will also be out of the plane, either above or below, in the complex with the *L. casei* enzyme. The 4′-methoxy carbon (compound B) gave rise to a single ^{13}C absorption in the spectrum of the enzyme–trimethoprim complex over the temperature range studied. Even at the lowest temperatures used (277 K), no evidence for two conformations could be detected, although one cannot exclude the possibility that the "up" and "down" orientations of the p-methoxy group have closely similar chemical shifts. If the benzyl ring does a 180° flip about its C1′–C4′ axis, then if the p-methoxy group is to maintain a preferred orientation relative to the enzyme, it must undergo an interconversion resulting in it moving to the opposite side of the ring: thus, the overall activation energy will have contributions from both dynamic processes. It should

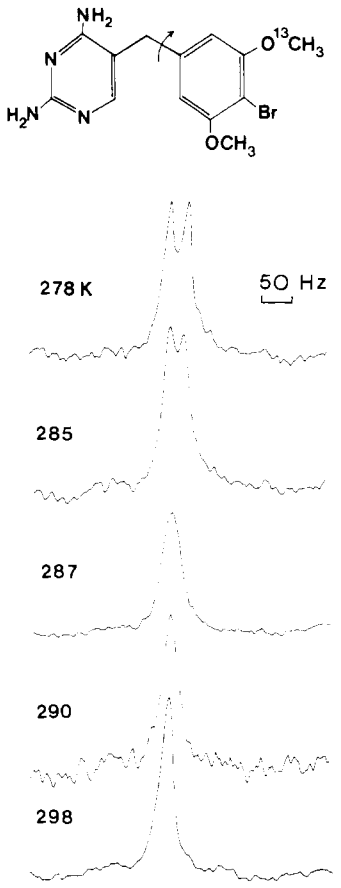


FIGURE 2: 50.2-MHz ^{13}C NMR spectra of the complex of [*m-methoxy*-^{13}C]brodimoprim with 1 equiv of *L. casei* dihydrofolate reductase at a series of temperatures.

be noted that interconversion between the two possible orientations of the *p*-methoxy group in the absence of ring flipping would not lead to averaging of the H2′ and H6′ chemical shift environments.

In order to examine the ring flipping without the complication of the dynamic process involving the *p*-methoxy group, we have examined the *p*-bromo analogue with ^{13}C labeling at the *m*-methoxy groups (compound E). Figure 2 shows the ^{13}C spectra of the [*m-methoxy*-^{13}C]brodimoprim (E) in its complex with dihydrofolate reductase at a series of temperature in the range 278 to 298 K. At 298 K, a single ^{13}C signal is detected for the 3′- and 5′-methoxy carbons. As the temperature is lowered, the signal broadens, and below 285 K, two separate signals of equal intensity are observed, which become narrower as the temperature is lowered further to 278 K. These spectra are characteristic of nuclei undergoing exchange between two equally populated magnetically nonequivalent sites. At low temperature, there are two nonequivalent environments for the *m*-methoxy ^{13}C nuclei at the C3′ and C5′ positions (see E_a and E_b): this would be expected if the benzyl ring was in a fixed orientation in its asymmetric binding site. Any exchange process that interchanges the environments of the 3′- and 5′-methoxy groups could lead to coalescence of the two signals. The simplest exchange process that can be envisaged in the present case is that resulting from motion about the C7–C1′ bond: such a rotation or flipping motion about the symmetry axis of the benzyl ring could average the environment of substituents at the C3′ and C5′ positions.

We have fitted the data in Figure 2 with line shapes calculated using the equations for exchange between two populated sites on the basis of McConnell's (1958) modifications of the Bloch equations. This allows one to estimate the rate of the "flipping" process and the activation parameters. At 287 K, the flipping rate constant is 65 ± 8 s^{-1} and E_A 132 ± 15 kJ/mol. Dynamic processes of this type have been described previously for motions about the symmetry axis of the aromatic rings of phenylalanine and tyrosine residues in several proteins (Campbell et al., 1975, 1976; Snyder et al., 1975; Wuthrich & Wagner, 1975; Wagner, 1980; Karplus & McCammon, 1981) and also in ligands bound to proteins (Cayley et al., 1979; Feeney et al., 1981; Clore et al., 1984). Although the NMR experiments cannot distinguish between a flipping mechanism (180° jumps about a symmetry axis) and free rotation, the former mechanism is more probable (Karplus and McCammon, 1981; Gall et al., 1982; Wagner, 1983).

A consideration of the structures of the complex of brodimoprim (and trimethoprim) with the enzyme reveals that in the bound conformation of the ligand (Birdsall et al., 1983) it would be impossible to flip the benzyl ring by 180° about the C7–C1′ bond because of steric interactions between the pyrimidine H6 and benzyl H2′ and H6′ protons. Flipping can only be achieved if the θ_1 torsion angle is changed by at least 60°. Since we know that ring flipping occurs in complexes of the enzyme with brodimoprim (and trimethoprim), then we must postulate that the structure of the complex fluctuates such that a conformation momentarily exists with a θ_1 angle that allows flipping to be accomplished without steric interactions between the pyrimidine and benzyl ring. The fluctuations in the structure of the complex will involve considerable movement of the benzyl ring and also of residues on the protein (such as Leu-19 and -27), which form part of its hydrophobic binding pocket.

We have also studied the effects of coenzyme binding on the ring flipping process. For the complex of the enzyme with [*m-methoxy*-^{13}C]brodimoprim and $NADP^+$, we observed spectra similar to those shown in Figure 2. Separate spectra for the two forms (I and II) (known to be present from ^{31}P NMR studies) are not resolved, probably because the lines are fairly broad. The coalescence temperature (287 K) was the same as that found for the binary complex, indicating a similar rate of flipping. At this temperature, the ternary complex is predominantly in form II (75% as estimated from ^{31}P spectra): this form is the one that most closely resembles the binary form, and it is interesting that this similarity extends to the flipping behavior of the benzyl ring.

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