Trimethoprim Binding to Bacterial and Mammalian Dihydrofolate Reductase: A Comparison by Proton and Carbon-13 Nuclear Magnetic Resonance†

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ABSTRACT: The binding of trimethoprim to dihydrofolate reductase from L1210 mouse lymphoma cells has been studied by measuring the changes in chemical shift of nuclei of the ligand that accompanying binding. The 6- and 2′,6′-proton chemical shifts of bound trimethoprim have been determined by transfer of saturation experiments, and the 2-carbon chemical shift has been determined by using [2-^{13}C]trimethoprim. The changes in proton chemical shift are substantially smaller than those accompanying binding to bacterial dihydrofolate reductase [Cayley, P. J., Albrand, J. P., Feeney, J., Roberts, G. C. K., Piper, E. A., & Burgen, A. S. V. (1979) *Biochemistry 18*, 3886]. It is shown that this difference arises largely from the fact that trimethoprim adopts different conformations when bound to mammalian and to bacterial dihydrofolate reductase. The proton chemical shifts are interpreted in terms of ring-current contributions from the two aromatic rings of trimethoprim itself and the nearby aromatic amino acid residues of the enzyme. The latter have been located by using the refined crystallographic coordinates of the *Lactobacillus casei* and *Escherichia coli* reductases in their complexes with methotrexate [Bolin, J. T., Filman, D. J., Matthews, D. A., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13650], under the assumption that, as indicated by the ^{13}C chemical shifts, the diaminopyrimidine ring of trimethoprim binds in the same way as does the corresponding part of methotrexate. With use of these assumptions, the conformation of trimethoprim bound to the dihydrofolate reductases from *L. casei*, *E. coli*, and L1210 cells has been calculated. Support for the calculated conformations has been obtained from the observation of transferred nuclear Overhauser effects between the 6- and 2′,6′-protons. The calculated conformations of trimethoprim bound to the bacterial and mammalian enzymes differ significantly, by ~25° in each of the two torsion angles, leading to a quite different overall shape for the molecule. These results are compared to the available crystallographic information, and the origins of the difference in conformation and its implications for understanding the selectivity of trimethoprim are briefly discussed.

The antibacterial drug trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] acts by inhibiting the enzyme dihydrofolate reductase (Roth et al., 1962; Burchall & Hitchings, 1965; Bushby & Hitchings, 1968). It owes its clinical usefulness [see, for example, Finland & Kass (1973) and Hitchings (1983)] to the fact that it is a much more potent inhibitor of the bacterial than of the mammalian enzyme. For example, the concentration required to produce 50% inhibition of human dihydrofolate reductase is 30000–60000 times greater than that required to have the same effect on the enzyme from *Escherichia coli* (Burchall & Hitchings, 1965; Li et al., 1982). [As pointed out by Baccanari et al. (1982), the K_m for dihydrofolate is generally about 10-fold lower with the mammalian than with the bacterial enzyme, so the ratio of K_i values for trimethoprim is of the order of 3000.]

In order to understand this selectivity in structural terms, it is important to compare the mode of binding of trimethoprim to bacterial and mammalian dihydrofolate reductase. We have earlier described the use of proton nuclear magnetic resonance (^{1}H NMR) and ring current shift calculations to determine the conformation of trimethoprim bound to the dihydrofolate reductase from *Lactobacillus casei* and *E. coli* (Cayley et al., 1979). Two possible conformations for the bound drug were deduced from these experiments, and one was subsequently found to correspond closely to the conformation observed in crystals of the *E. coli* enzyme–trimethoprim complex (Baker et al., 1981).

We have now carried out similar experiments using dihydrofolate reductase isolated from the mouse L1210 lymphoma cell line. In the present paper, we report calculations of the conformation of trimethoprim bound to this enzyme, as well as a refinement of the corresponding calculations for the complexes with the *L. casei* and *E. coli* enzymes. We show that trimethoprim has a different conformation when bound to mammalian as compared to bacterial dihydrofolate reductase.

Materials and Methods

[2-^{13}C]Trimethoprim was a generous gift from Dr. S. Daluge, Wellcome Research Laboratories, Research Triangle Park, NC 27709 [see Roberts et al. (1981)]. 2,4-Diaminopyrimidine was obtained from Bachem, Inc.

Purification of Dihydrofolate Reductase from L1210 Cells. The enzyme was isolated from a methotrexate-resistant line of mouse L1210 (lymphoblastoid) cells in which dihydrofolate reductase represents about 1% of the total soluble protein. The cells were grown in vitro in suspension culture in RPMI 1640 medium on a scale of 10–15 L/week. Full details of the purification of the enzyme will be published elsewhere (J. G. Dann, unpublished results); briefly, the procedure was as follows. The crude cell extract (~5 × 10^9 cells, 300 mL) was fractionated by gel filtration on Sephadex G-75 (80 × 10 cm) in 50 mM potassium phosphate–100 mM KCl, pH 6.5. Fractions containing dihydrofolate reductase activity were pooled and loaded on to an affinity column of methotrexate–aminohexyl-Sepharose [6 × 1.6 cm; prepared according to Dann et al. (1976)] equilibrated in the same buffer. The

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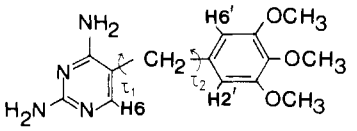

FIGURE 1: Structure of trimethoprim. The two torsion angles were defined by the atoms C4–C5–C7–C1′ (τ_1) and C5–C7–C1′–C2′ (τ_2); the torsion angles are zero when atoms α–β–γ–δ are syn-planar, and a positive rotation is one that moves atom δ in a clockwise sense when one looks along the β–γ bond from β to γ.

column was washed with 50 mM potassium phosphate–2 M KCl, pH 7.0, until the absorbance of the effluent at 280 nm was less than 0.02. Dihydrofolate reductase was eluted with 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 2 M KCl, and 2 mM folate, pH 8.5. Folate was removed from the enzyme by passage down a column (90 × 2.6 cm) of Sephadex G-25 equilibrated with 50 mM Tris-HCl–2 M KCl, pH 8.5, and this buffer was exchange for 50 mM potassium phosphate–100 mM KCl, pH 6.5, on a second Sephadex G-25 column (30 × 2.6 cm). All operations were carried out at 4 °C.

The purified enzyme was free from contamination by nucleotide or residual folate, having λ_{max} at 281 nm and $A_{330} < 0.01\ A_{281}$. Analytical isoelectric focusing revealed the presence of two components in the purified enzyme, the major (96–97%) component having p*I* 8.2 and the minor (3–4%) one p*I* 7.0. Both components were enzymatically active, with the same turnover number.

NMR Spectroscopy. 270-MHz ^{1}H NMR spectra were obtained by using a Bruker WH270 spectrometer. The samples contained 0.2 mM enzyme, 50 mM potassium phosphate (pH* 6.5), and 100 mM KCl in 0.4 mL of ^2H_2O. (The notation pH* indicates a pH meter reading uncorrected for the deuterium isotope effect on the glass electrode.) Saturation-transfer experiments were carried out at 285 K on a sample containing 1 mM trimethoprim, as described earlier (Cayley et al., 1979; Hyde et al., 1980). Chemical shifts are reported relative to dioxane (3.71 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate); downfield shifts are positive.

50.3-MHz ^{13}C NMR spectra were obtained by using a Bruker WM200 spectrometer. The sample contained 0.5 mM enzyme, 0.45 mM [2-^{13}C]trimethoprim, 50 mM phosphate (pH* 6.5), and 100 mM KCl in 3.0 mL of ^2H_2O. Spectra were obtained at a sample temperature of 285 K, with a pulse angle of 25°, a pulse interval of 0.82 s, and a spectral width of 10 kHz. Chemical shifts are reported relative to external dioxane (50% in ^2H_2O).

Calculation of Ring-Current Chemical Shifts. The Johnson-Bovey (1958) equation was used to calculate the shielding effects of aromatic rings of the protons of bound trimethoprim as a function of the two torsion angles τ_1 (C4–C5–C7–C1′) and τ_2 (C5–C7–C1′–C2′; see Figure 1). The trimethoprim molecule was constructed from standard bond lengths and angles, with the exception of the C5–C7–C1′ bond angle, which was set to 116° (Koetzle & Williams, 1976; E. Potterton, personal communication). The molecule was positioned so that its pyrimidine ring coincided with the corresponding part of the pteridine ring of methotrexate in either the *L. casei* enzyme–methotrexate–NADPH complex or the *E. coli* enzyme–methotrexate complex as determined by X-ray crystallography (Matthews et al., 1977, 1978; Bolin et al., 1982; refined coordinates kindly provided by Dr. D. A. Matthews). For calculations on the L1210 enzyme (for which only preliminary crystallographic information is available; Stammers et al., 1983), the *L. casei* structure was used, and Phe-31 (L1210; Stone et al., 1979) was constructed from the homologous Leu-27 (*L. casei*) as follows: the C_α, C_β, and C_γ coordinates were taken to be the same, and a planar aromatic ring was built (with standard bond lengths and angles) on to C_γ, by taking the C_γ–C_{δ_1} bond of the ring to lie along the C_γ–C_{δ_1} bond of Leu-27.

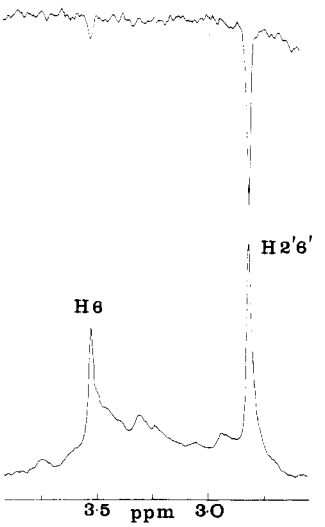

FIGURE 2: (Lower) Aromatic region of 270-MHz ^{1}H spectrum of a sample containing 1 mM trimethoprim and 0.2 mM L1210 dihydrofolate reductase. The aromatic proton resonances of the excess, free, trimethoprim are labeled. (Upper) Difference between this spectrum and one in which a saturating radio frequency field had been applied at 2.41 ppm, the resonance frequency of H2′,6′ in bound trimethoprim. The marked decrease in intensity of the H2′,6′ resonance of free trimethoprim arises from transfer of saturation and the smaller effect on the H6 resonance from a transferred NOE effect.

Results

^{1}H Chemical Shifts. The chemical shifts of the aromatic proton resonances of trimethoprim in its complex with L1210 dihydrofolate reductase have been determined by the saturation-transfer method (Forsén & Hoffman, 1963). In a sample containing 0.2 mM enzyme and 1 mM trimethoprim, the protons of trimethoprim exist in two environments, corresponding to the free and bound states; if the rate of exchange between these states is sufficiently slow, separate signals will be seen for each state. In the spectrum shown in Figure 2, the resonances from the excess free trimethoprim are clearly visible, while those from the bound ligand are not. If the resonance of a proton in the bound state is saturated, this saturation will be transferred to the free state by the exchange process, and a decrease in intensity of the corresponding resonance of free trimethoprim will be observed (provided that the exchange rate is at least comparable to the spin–lattice relaxation rate in the free state). Thus, as shown by the difference spectrum in Figure 2, irradiation at 2.41 ppm leads to a large decrease in intensity of the resonance of the 2′,6′-protons of free trimethoprim.

Systematic irradiation throughout the relevant region of the spectrum thus allows us to locate the resonances of bound trimethoprim. The results of such an experiment are shown in Figure 3. In addition to the direct effect of irradiation at the frequency of the free H2′,6′ resonance at 2.83 ppm, a substantial "peak" is observed centered on 2.41 ppm. This corresponds to transfer of saturation from the corresponding signal of bound trimethoprim, which must therefore have a chemical shift of 2.41 (±0.04) ppm. The H2′,6′ resonance thus shifts upfield by 0.42 ppm on binding to the L1210 enzyme; this is only about half the change in chemical shift seen on binding to the *L. casei* enzyme (Cayley et al., 1979). The

Table I: Aromatic ^{1}H Chemical Shifts of Trimethoprim and 2,4-Diaminopyrimidine Bound to Dihydrofolate Reductase from L1210 Cells, *L. casei*, and *E. coli*

	change in chemical shift (ppm)[a]			
	L1210	*L. casei*[b]	*E. coli* I[b]	*E. coli* II[b]
trimethoprim				
H6	−0.33 (±0.08)	−1.10 (±0.04)	−1.26 (±0.04)	−1.07 (±0.04)
H2′,6′	−0.29 (±0.04)	−0.60 (±0.04)	−0.57 (±0.04)	−0.48 (±0.04)
2,4-diaminopyrimidine				
H6	−0.35 (±0.07)	−0.23 (±0.07)[c]	−0.18 (±0.07)[d]	

[a] For trimethoprim, expressed relative to 2,4-diamino-5-methylpyrimidine (H6) or 1-alkyl-3,4,5-trimethoxybenzene (H2′,6′) to correct for the averaged ring-current effects of the pyrimidine ring on H2′,6′ and of the benzyl ring on H6 in free trimethoprim; see Cayley et al. (1979). For 2,4-diaminopyrimidine, expressed relative to free 2,4-diaminopyrimidine. Negative shifts are upfield. [b] From Cayley et al. (1979). *E. coli* isoenzymes I and II differ only in residue 28, which is Leu in I and Arg in II (Baccanari et al., 1981). [c] From Feeney et al. (1977). [d] Measured with a mixture of isoenzymes I and II of the *E. coli* enzyme; Cayley (1980).

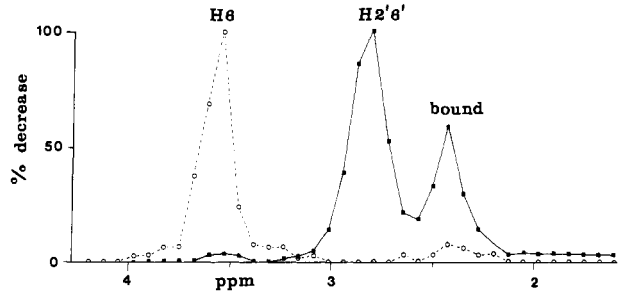

FIGURE 3: Effects of applying a saturating radio-frequency field at various frequencies in the aromatic region of the spectrum on the intensities of the aromatic proton resonances of free trimethoprim in a sample containing 1 mM trimethoprim and 0.2 mM L1210 dihydrofolate reductase. The decrease in intensity of the H6 (○) and H2′,6′ (■) resonances is shown as a function of the frequency of the saturating field (ppm from dioxane). The "peaks" labeled H6 and H2′,6′ correspond to direct saturation of the free-ligand resonances.

fact that only a single transfer of saturation peak is seen in Figure 3 for the 2′- and 6′-protons strongly suggests that, as previously observed with the bacterial enzyme (Cayley et al., 1979), the benzyl ring of bound trimethoprim is "flipping" about its symmetry axis (C1′–C4′) sufficiently rapidly to average the chemical shifts of these two protons.

In the case of the H6 resonance, however, only the peak corresponding to direct saturation of the signal of the free ligand is observed (Figure 3). The exchange rate must obviously be the same for this proton as for the 2′,6′-protons, and its spin–lattice relaxation time in the free state is somewhat longer than that of H2′,6′, so that one would expect that the magnitude of the transfer of saturation effect would be at least as great for H6 as for H2′,6′. Since no such effect is observed, one can only conclude that the H6 resonance of bound trimethoprim is within 0.05 ppm of the corresponding resonance of the free compound (some further evidence for this is presented below). This is in marked contrast to the shift of 0.77 ppm upfield from the resonance of free trimethoprim seen with the *L. casei* enzyme (Cayley et al., 1979).

In solution, free trimethoprim exists as a mixture of many rapidly interconverting conformations about the C5–C7 and C7–C1′ bonds (Koetzle & Williams, 1976), so the observed chemical shift of H6 will contain an averaged contribution from the ring current of the benzyl ring, and that of H2′,6′ will contain a contribution from the ring current of the pyrimidine ring. Free trimethoprim is thus not a suitable reference compound if the chemical shifts in the complex are to be interpreted in terms of ring-current shifts (see Discussion). Therefore, in the summary in Table I of the chemical shifts of trimethoprim bound to dihydrofolate reductase from L1210 cells, *L. casei*, and *E. coli*, we have expressed the shifts relative to the model compounds 2,4-diamino-5-methylpyrimidine (for H6) and 1-alkyl-3,4,5-trimethoxybenzene (for H2′,6′); the chemical shifts in these model compounds have no contributions from conformation-dependent ring-current effects.

Scheme I: Pathways of Magnetization Transfer Involved in the Transferred Nuclear Overhauser Effect

As an aid to the analysis of the trimethoprim chemical shifts, we have also measured the change in chemical shift of the H6 resonance of 2,4-diaminopyrimidine on binding to the L1210 enzyme. This compound binds relatively weakly to the enzyme, so that there is fast exchange between the bound and free states, and a single average resonance is observed. Analysis of the concentration dependence of the chemical shift of this resonance gave a dissociation constant of 0.5 (±0.15) × 10^{-3} M and a chemical shift in the bound state of 0.35 (±0.07) ppm upfield from free 2,4-diaminopyrimidine.

Transferred Nuclear Overhauser Effect. In the difference spectrum in Figure 2, it is evident that irradiation at 2.41 ppm leads to a small decrease in the intensity of the H6 resonance of free trimethoprim as well as the large decrease in intensity of the H2′,6′ resonance due to transfer of saturation. This small effect on H6 arises from a transferred nuclear Overhauser effect (Albrand et al., 1979; Cayley et al., 1979; Hyde et al., 1980; Feeney et al., 1983). The observation of a nuclear Overhauser effect (NOE) between two nuclei indicates that there is cross relaxation between them and therefore, provided that the effect is specific and not simply due to spin diffusion, that they are close together in space (Noggle & Schirmer, 1971). Thus, with reference to Scheme I, irradiation at the resonance frequency of H2′,6′ of bound trimethoprim leads to a decrease in the intensity of the H6 resonance of the bound ligand by cross relaxation, and this effect is transferred to the H6 signal of free trimethoprim (as seen in Figure 2) by the exchange process—the transferred nuclear Overhauser effect (TNOE). It is apparent from Figure 3 that this is a very specific effect, being seen only on irradiation within a narrow frequency range around 2.41 ppm. In addition, since the sign of the NOE depends on the correlation time of the internuclear vector (Noggle & Schirmer, 1971), the observation of a negative TNOE (a decrease in intensity) demonstrates that the effect originates from the bound ligand. It follows that the conformation of trimethoprim bound to L1210 dihydrofolate reductase is such that H6 is close to H2′ and/or H6′; this is also the case for the complex with the *L. casei* enzyme (Cayley et al., 1979). No absolute distance measurement can be made, but it has been estimated that a significant TNOE

implies an internuclear distance of less than 4 Å (Albrand et al., 1979).

It can be seen from Figure 3 that there is also a small but significant decrease in intensity of the H2′,6′ resonance of free trimethoprim on irradiation at 3.52 ppm, close to the position of the H6 resonance of the free ligand. As noted above, such a decrease in intensity must arise from an NOE in the bound ligand, i.e., from a TNOE. If the irradiation at 3.52 ppm is saturating only the resonance of H6 in free trimethoprim, then, with reference to Scheme I, this TNOE must arise by the following pathway:

$$H6_F \rightarrow H6_B \rightarrow H2',6'_B \rightarrow H2',6'_F$$

However, as noted above, in free trimethoprim the relaxation rate of H6 is slower than that of H2′,6′, so that one would expect to observe a TNOE by the reverse of this pathway—a decrease in the H6 signal of the free ligand on irradiation of its H2′,6′ signal; this is not observed (Figure 3). Alternatively, if, as suggested above, the H6 resonance of bound trimethoprim is very close to the corresponding resonance from the free ligand, irradiation at 3.52 ppm would saturate the resonance of the bound ligand directly. The observed TNOE would then arise from the pathway $H6_B \rightarrow H2',6'_B \rightarrow H2',6'_F$, exactly the reverse of the TNOE seen on irradiation at 2.41 ppm and discussed above. Since it involves fewer steps at which spin–lattice relaxation can compete with the transmission of the TNOE, this path should give a larger effect than that from $H6_F$ to $H2',6'_F$. This experiment thus confirms that there is very little change in the chemical shift of the H6 resonance of trimethoprim when it binds to L1210 dihydrofolate reductase.

^{13}C Chemical Shifts. The ^{13}C chemical shift of the 2-carbon resonance of trimethoprim has been measured by using [2-^{13}C]trimethoprim under conditions where the enzyme concentration was higher than the ligand concentration, to ensure that all the trimethoprim was bound. The chemical shift of this resonance is 89.30 ppm for trimethoprim bound to the L1210 enzyme. This is the same, within experimental error, as the value of 89.26 ppm observed for trimethoprim bound to the *L. casei* enzyme (Roberts et al., 1981).

Discussion

A qualitative comparison of the mode of binding of trimethoprim to the dihydrofolate reductase from L1210 cells and *L. casei* can readily be made by inspection of the ^1H and ^{13}C chemical shift changes that accompany binding.

First, the 2-^{13}C chemical shift of trimethoprim is the same in both complexes. The shift of this resonance is very sensitive to the protonation state of the pyrimidine ring (differing by 7.09 ppm between protonated and unprotonated trimethoprim; Roberts et al., 1981). The similarity of the shift in the complex (89.30 ppm) to that of free trimethoprim protonated on N1 (88.0 ppm) therefore indicates that trimethoprim must also be protonated on N1 when bound to L1210 dihydrofolate reductase, as it is when bound to the *L. casei* (Roberts et al., 1981; A. W. Bevan, G. C. K. Roberts, J. Feeney, and L. Kuyper, unpublished results) and *Streptococcus faecium* (Blakley et al., 1983) enzymes. The relatively small difference in chemical shift between free and bound protonated trimethoprim (1.30 ppm) reflects an additional shielding contribution arising from the environment of the protonated trimethoprim in the binding site (Roberts et al., 1981), which is the same in both complexes.

The ^{13}C evidence thus suggests that the mode of binding of the pyrimidine ring of trimethoprim to the bacterial and mammalian dihydrofolate reductase is very similar. However, the ^1H chemical shift changes observed in trimethoprim on binding to the L1210 enzyme are much smaller than those accompanying binding to the *L. casei* or *E. coli* enzymes. The chemical shift of the H6 resonance of bound trimethoprim (Table I) is made up of two components: one arising from the effects of amino acid residues in or near the binding site and the other, the "internal" component, from the ring current of the trimethoxybenzyl ring. The latter will obviously depend on the conformation of the bound trimethoprim molecule. If we assume that 2,4-diaminopyrimidine binds in the same way as the pyrimidine ring of trimethoprim, we can use its H6 shift as a measure of the effects of nearby amino acid residues. In this way can estimate the internal, conformation-dependent contribution to the H6 shift of trimethoprim as −0.87 ppm in the complex with the *L. casei* enzyme but only 0.02 ppm in the complex with the L1210 enzyme. It is thus clear that there is a difference in the conformation of trimethoprim between the bacterial and mammalian complexes.

In order to arrive at a quantitative description of this difference in conformation, we need to be able to calculate the H6 and H2′,6′ chemical shifts as a function of the trimethoprim conformation. This requires that we make two assumptions: (i) We assume that those amino acid residues that perturb the chemical shift of the trimethoprim protons have the same spatial relationship to the pyrimidine ring of trimethoprim as they do to the corresponding part of methotrexate in the refined crystal structures of the *L. casei* enzyme–methotrexate–NADPH and *E. coli* enzyme–methotrexate complexes (Bolin et al., 1982). In the bacterial enzymes there is now good evidence for closely similar binding of the pyrimidine ring of trimethoprim and the corresponding part of methotrexate: for the *L. casei* enzyme from NMR (Cocco et al., 1981; Roberts et al., 1981) and for the *E. coli* enzyme from crystallography (Matthews et al., 1977; Baker et al., 1981; Bolin et al., 1982). (ii) We assume that the observed chemical shift effects on the protons of trimethoprim arise solely from the magnetic anisotropy shielding effects of nearby aromatic rings. In the bacterial enzyme, only one aromatic amino acid residue, Phe-30 (*L. casei* numbering), is close enough to the bound ligand to have a significant effect. In the L1210 enzyme (Stone et al., 1979), the homologous residue is Tyr-34, and in addition Phe-31 (homologous to Leu-27) must be considered. Thus, assumption i involves only these two residues.

Any conformation that gave calculated chemical shifts in agreement with experimental values but involved unfavorable steric interactions *within* the trimethoprim molecule was eliminated from further consideration.

Conformation of Trimethoprim Bound to Bacterial Enzyme. With the availability of a structure refined at 1.7-Å resolution (Bolin et al., 1982; D. A. Matthews, personal communication), we are now able correspondingly to refine our previous calculations (Cayley et al., 1979) of the conformation of trimethoprim bound to the bacterial enzyme. The contribution of Phe-30 to the H6 chemical shift will be independent of the trimethoprim conformation; it is calculated, from the refined crystallographic coordinates of the *L. casei* enzyme–methotrexate–NADPH complex, to be −0.19 ppm (upfield shifts negative).

Taking the observed H6 chemical shift for trimethoprim bound to the *L. casei* enzyme and subtracting this calculated value, we obtain −0.91 ppm as the contribution from the benzyl ring. We have calculated the ring-current effect on the H6 chemical shift due to the benzyl ring as a function of the two dihedral angles, τ_1 and τ_2 (defined in Figure 1), and we find

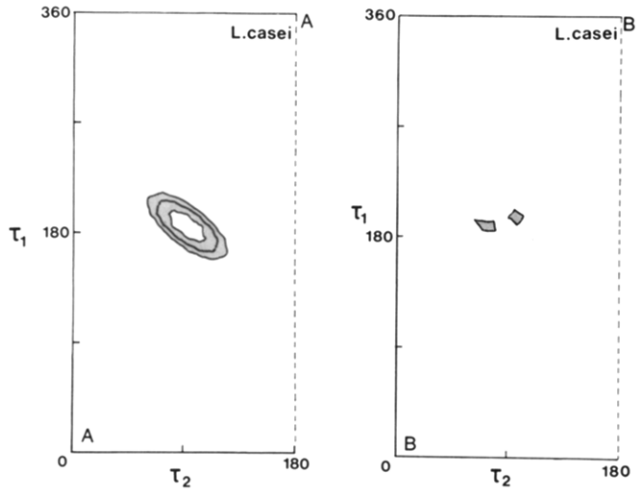

FIGURE 4: Calculation of conformation of trimethoprim bound to *L. casei* dihydrofolate reductase. (A) The contribution to the H6 chemical shift from the ring-current effect of the benzyl ring as a function of the torsion angles τ_1 and τ_2. The shaded area indicates the region of conformational space in which the calculated chemical shift contribution is within ±0.1 ppm of the experimental estimate (−0.91 ppm; central contour). (B) The regions of conformational space in which the calculated total H6 and H2′,6′ chemical shifts are simultaneously within ±0.1 ppm of the experimental estimates for the *L. casei* enzyme (Table I). The two shaded areas correspond to solutions LCl and LC2 (Table II). For details of the calculations, see the text.

that an effect of the required magnitude (−0.9 ± 0.1 ppm) is only obtained in a small region of conformational space indicated by the shaded area in Figure 4A.

The range of possible conformations for trimethoprim bound to the *L. casei* enzyme can be narrowed down further by taking into account the H2′,6′ chemical shift. A full calculation, incorporating both the internal ring-current effects and those of Phe-30 on both H6 and H2′,6′ shifts, led to two rather closely defined conformational solutions, shown as the shaded areas in Figure 4B. These solutions, denoted LC1 and LC2 (see Table II), are centered on τ_1, τ_2 values of 191°, 93° and 184°, 70°, respectively; they both give calculated chemical shifts (H6, −1.11 ppm; H2′,6′, −0.60 ppm) essentially identical with the experimental values.

Similar calculations have been carried out for the *E. coli* enzyme by using the chemical shifts observed with both isoenzymes (Table I) and the refined coordinates of both molecules in the unit cell of the *E. coli* enzyme–methotrexate complex (Bolin et al., 1982). In each case, either one or two conformational solutions were obtained, each as closely defined as those for the *L. casei* enzyme shown in Figure 4B. These solutions are summarized in Table II. All the possible solutions for trimethoprim bound to the bacterial enzymes are seen to be similar, with τ_1 values such that the benzyl ring is folded down next to the pyrimidine ring and τ_2 values such that the two rings are almost at right angles. The 6-proton is close to one of the ortho protons on the benzyl ring in all these conformations, consistent with the observation of a TNOE between them (Cayley et al., 1979).

It is difficult to estimate the likely precision of these conformational solutions. From consideration of the errors involved in the chemical shift measurements, in the atomic positions determined crystallographically, and in the estimation of ring-current effects by the Johnson–Bovey equation, we estimate that the precision of the calculated conformations is about ±10°. On this basis, there appears to be no significant difference between the conformations of trimethoprim bound to the three bacterial enzymes. Of the two possible solutions

Table II: Possible Conformations of Trimethoprim Bound to Bacterial Dihydrofolate Reductase[a]

conformational solution	enzyme[b]	coordinates[c]	τ_1 (deg)	τ_2 (deg)
LC1	*L. casei*	*L. casei*	191	93
LC2	*L. casei*	*L. casei*	184	70
EC I	*E. coli* I	*E. coli* 1	193	75
		E. coli 2	190	72
EC II 1	*E. coli* II	*E. coli* 1	200	88
		E. coli 2	201	85
EC II 2	*E. coli* II	*E. coli* 1	188	61
		E. coli 2	186	62

[a] Derived from ^{1}H NMR data as described in the text. [b] Enzyme on which NMR data were obtained; *E. coli* I and II denote isoenzymes I and II from *E. coli*. [c] Crystallographic coordinates used to define the position of Phe-30. *L. casei* denotes the *L. casei* enzyme–methotrexate–NADPH complex. *E. coli* 1 and *E. coli* 2 denote molecules 1 and 2 of the *E. coli* enzyme–methotrexate complex.

for the complex with the *L. casei* enzyme, LC2 is clearly closer to the single solution, EC 1 for *E. coli* isoenzyme I, and solutions LC2, EC I, EC II 1, and EC II 2 are all within ±13° of the "consensus solution" τ_1 = 191° and τ_2 = 73°.

In the crystal structure of the *E. coli* enzyme–trimethoprim complex (Baker et al., 1981), the trimethoprim molecule has τ_1 = 177° and τ_2 = 76° (based on the refined structure; C. R. Beddell and D. A. Matthews, personal communication). The difference in τ_1 and τ_2 values from those calculated here for *E. coli* isoenzyme I is no more than 15° and well within the combined error of the crystallographic and NMR measurements. We conclude that the conformation of trimethoprim bound to dihydrofolate reductase is the same in solution as in the crystal and is the same for the *L. casei* enzyme and for both isoenzymes from *E. coli*.

Conformation of Trimethoprim Bound to L1210 Enzyme. The basic procedure employed to calculate the conformation of trimethoprim bound to the mammalian enzyme is identical with that outlined above, except that the effects of an additional aromatic ring, Phe-31 (homologous to Leu 27), must be considered. As discussed above, comparison of the H6 chemical shifts in trimethoprim and in 2,4-diaminopyrimidine bound to the L1210 enzyme shows that the internal contribution to the shift of this proton is very small (ca. 0.02 ppm). The shaded area in Figure 5A indicates those conformations in which the contribution of the ring current of the benzyl ring to the H6 chemical shift is 0 ± 0.1 ppm. The absence of a significant internal ring-current shift shows that the consensus solution obtained for the bacterial enzyme is not a possible conformation for trimethoprim bound to the L1210 enzyme.

From assumptions i and ii above, the H2′,6′ chemical shift will be determined by the ring-current effects of Phe-31, Tyr-34, and the pyrmidine ring. We assume that the geometrical relationship between Tyr-34 and the pyrimidine ring is exactly the same as that between the homologous Phe-30 and the pyrimidine ring in the *L. casei* enzyme complex, but the orientation of the aromatic ring of Phe-31 remains to be established. The possible positions of this ring can be calculated by considering the H6 chemical shift of 2,4-diaminopyrimidine bound to the L1210 enzyme. The observed shift is 0.35 ppm, and the contribution from Tyr 34 (identical with that from Phe-30 of the *L. casei* enzyme) is −0.19 ppm, leaving −0.16 ppm as the contribution of Phe-31. This can be used to define the possible orientations of this phenylalanine ring. Continuing to base our calculations on the refined crystallographic coordinates of the *L. casei* enzyme (Bolin et al., 1982),

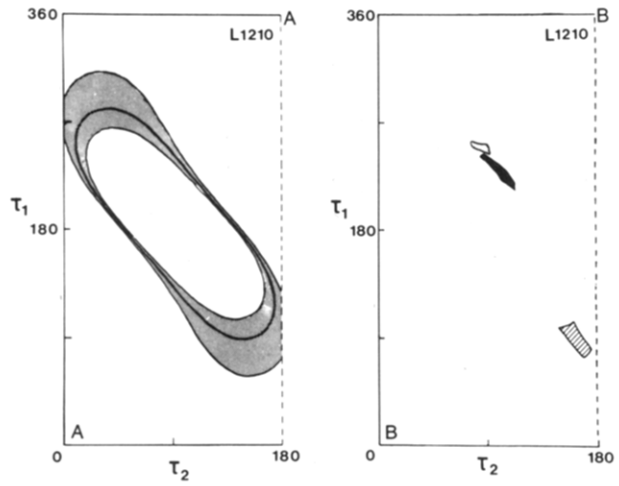

FIGURE 5: Calculation of conformation of trimethoprim bound to L1210 dihydrofolate reductase. (A) The contribution to the H6 chemical shift from the ring-current effect of the benzyl ring as a function of the torsion angles τ_1 and τ_2. The shaded area indicates the region of conformational space in which the calculated chemical shift is within ±0.1 ppm of the experimental estimate (0.02 ppm; central contour). (B) The regions of conformational space in which the calculated total H6 and H2′,6′ chemical shifts are simultaneously within ±0.1 ppm of the experimental estimates for the L1210 enzyme (Table I). The shaded area corresponds to solution MB1, the hatched area to solution MA1, and the black area to solution MA2 (Table IV). For details of the calculations, see the text.

Table III: Possible Conformations of Trimethoprim Bound to L1210 Dihydrofolate Reductase[a]

conformational solution[b]	τ_1 (deg)	τ_2 (deg)	comments[c]
MA1	90	170	distance H6 to H2′ or H6′ ⩾ 4 Å
MA2	228	110	
MB1	252	91	distance H6 to H2′ or H6′ ⩾ 4 Å; bad contact with Tyr-34

[a] Derived from ^{1}H NMR data as described in the text. [b] Solutions MA1 and MA2 have Phe-31 $\tau_{\beta\gamma} = 30°$; solution MB1 has Phe-31 $\tau_{\beta\gamma} = 110°$. [c] See text.

we "constructed" Phe-31 (L1210) from Leu-27 (*L. casei*) as described under Materials and Methods. With the pyrimidine ring positioned according to assumption i, the H6 chemical shift was calculated as a function of $\tau_{\beta\gamma}$, the torsion angle[1] about the C_β–C_γ bond of Phe-31. Two values of $\tau_{\beta\gamma}$ were found to give the required shift contribution of −0.16 (±0.1) ppm, namely, $\tau_{\beta\gamma} = 30°$ (±5°) and $\tau_{\beta\gamma} = 110°$ (±5°).

Taking each of these possible orientations of Phe-31 in turn, we calculated the H6 and H2′,6′ chemical shifts as a function of τ_1 and τ_2, incorporating the effects of the pyrimidine and benzyl rings of trimethoprim and the phenyl rings of Phe-31 and Tyr-34. A total of three possible conformational solutions was obtained,[2] as shown in Figure 5B; their dihedral angles are summarized in Table III.

It is possible to eliminate two of these solutions from further consideration, since, as noted above, a TNOE is observed between H6 and H2′,6′ for trimethoprim bound to the L1210 enzyme, implying that the distance between H6 and H2′ or H6′ is likely to be appreciably less than 4 Å (Albrand et al., 1979). However, in the conformations corresponding to solutions MA1 and MB1, neither H2′ nor H6′ is within 4 Å of H6. (In addition, in MB1 the benzyl ring makes a bad contact with the aromatic ring of Tyr-34.) The only one of the three solutions that remains is MA2, which is thus the most probable conformation for trimethoprim bound to L1210 dihydrofolate reductase. It gives calculated chemical shifts (H6, −0.39 ppm; H2′,6′, −0.28 ppm) identical (within experimental error) with the measured shifts. Solution MA2 is less closely defined than, for example, solution LC2 for the *L. casei* enzyme. Overall, the uncertainty is about ±17° in both τ_1 and τ_2, although the "family" of solutions represented by MA2 occupies a smaller region of conformational space than this implies, since the possible values of τ_1 and τ_2 are highly correlated (see Figure 5B). This conformation, derived from data obtained from the mouse (L1210) enzyme–trimethoprim binary complex in solution, is similar to that observed by Matthews et al. (1983) in crystals of the chicken liver enzyme–trimethoprim–NADPH ternary complex. In this respect, as in its amino acid sequence (Kumar et al., 1980; Volz et al., 1982), the avian enzyme appears to resemble the mammalian enzyme closely.

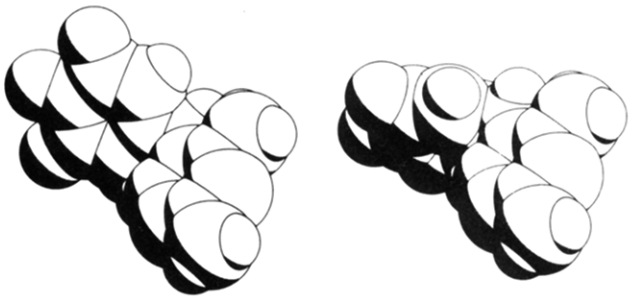

FIGURE 6: Comparison of space-filling representations of conformations of trimethoprim when bound to mammalian (left) and bacterial (right) enzymes. The two structures are shown with the diaminopyrimidine ring (bottom right in each structure) in exactly the same orientation.

Comparison of Trimethoprim Binding to Bacterial and Mammalian Enzyme. Thus the most probable conformations of trimethoprim in its binary complexes with the bacterial and the mammalian reductase in solution are described by τ_1, τ_2 values of 191°, 73° and 218°, 100°, respectively. These relatively modest changes of ~25° in dihedral angles make a very significant difference to the overall shape of the molecule, as illustrated by the "space-filling" models in Figure 6.

If one attempts to place trimethoprim in the conformation (MA2) characteristic of its complex with the L1210 enzyme into the binding site of the *E. coli* or *L. casei* enzyme, as defined by the refined crystal structures (Bolin et al., 1982), unfavorable steric interactions with residues 50 and 54 (*L. casei* numbering) in the loop between helix C and β-strand C are evident. In the eukaryotic enzymes, there is an insertion of two residues (relative to the *L. casei* sequence) in this loop, which, in the chicken liver enzyme (Volz et al., 1982), leads to a change in the loop conformation such that it moves away from the trimethoprim molecule, allowing the latter to bind in a conformation closely similar to MA2 (Matthews et al., 1983). The converse question, why trimethoprim does not bind to the mammalian enzyme in conformation LC2, is less easily answered; there appears to be no simple steric hindrance to this mode of binding. It seems likely that the trimethoxybenzyl ring is able to make more favorable hydrophobic contacts with the mammalian enzyme when trimethoprim is in conformation MA2 than when it is in conformation LC2. If this is the origin

[1] The dihedral angle $\tau_{\beta\gamma}$ is defined by C_α–C_β–C_γ–C_{δ_1}, such that the conformation of Leu-27 in the crystal has $\tau_{\beta\gamma} = 0°$. With reference to C_α–C_β–C_γ–C_{δ_1} syn-planar as $\tau_{\beta\gamma} = 0°$, the two orientations consistent with the 2,4-diaminopyrimidine chemical shift have $\tau_{\beta\gamma} = 206$ and 286°.

[2] We have also performed calculations of the H6 and H2′,6′ chemical shifts in which rotation about both C_α–C_β and C_β–C_γ was permitted. This gave a substantially larger number of orientations of Phe-31 that were consistent with the 2,4-diaminopyrimidine chemical shift but no additional conformational solutions for trimethoprim.

of the difference in mode of binding, then it may depend on more subtle difference in structure between the prokaryotic and eukaryotic enzymes, some of which are discusssed by Matthews et al. (1983).

As noted in the introduction, trimethoprim is a much more potent inhibitor of bacterial than of mammalian reductase. In attempting to explain this selectivity, it is obviously important to take account of the difference in conformation of the bound inhibitor. This will contribute to the selectivity in two ways. First, the conformational energy calculations of Koetzle & Williams (1976) suggest that conformation MA2 is intrinsically of somewhat higher energy than conformation LC2. Thus, a larger proportion of the "intrinsic" binding energy (Jencks, 1975) will be used up in changing the ligand conformation when trimethoprim binds to the mammalian enzyme than when it binds to the bacterial enzyme. Second, and probably more important, the trimethoxybenzyl ring must occupy a significantly different part of the binding site in the two different enzymes. The importance of the benzyl ring to the selectivity of trimethoprim is illustrated by the finding that the binding energies of 2,4-diaminopyrimidine to the L1210 enzyme, 4.5 (±0.2) kcal/mol, and to the *L. casei* enzyme, 4.2 (±0.07) kcal/mol (Birdsall et al., 1978, 1980), are very similar, and it is well-known (Baccanari et al., 1982; Hyde & Roth, 1982; Roth, 1983, and references cited therein) that the methoxy substituents on this ring are particularly crucial.

Analyses of the relationship between structure and inhibitory potency (determined as K_i values and, hence, in the presence of NADPH) among trimethoprim analogues in terms of substituent contribution (Dietrich et al., 1980; Smith et al., 1982; Li et al., 1981, 1982, and references cited therein; Hyde & Roth, 1982) have shown quite different patterns for the prokaryotic and eukaryotic enzymes. In the latter case, the dominant factor is found to be the hydrophobicity of the meta substituents, whereas for the *L. casei* and *E. coli* enzymes the molar refractivity of these substituents, which has been shown to be related to their specific space-filling characteristics (Hyde & Roth, 1982), is much more important. With the knowledge of the conformation of trimethoprim (and, presumably, its analogues) in complexes with the two groups of enzymes, it is clear that these differences in behavior reflect not simply differences in the amino acid residues lining a "fixed" binding site but a quite different orientation of the benzyl ring within the site. This illustrates the importance of detailed structural information to the interpretation of structure–activity relationships and to drug design. In this context, the observation of this conformation difference obviously suggests that conformationally restricted analogues would afford a useful further approach to the design of highly selective inhibitors. Thus, 5-phenyl-substituted 2,4-diamino-*s*-triazines and 2,4-diaminopyrimidines (such as the antimalarial drug pyrimethamine) cannot adopt a conformation in which they have a similar shape to that of trimethoprim in conformation LC2, whereas they an readily adopt a conformation resembling conformation MA2 of trimethoprim. It is indeed found that compounds of these classes are much more potent inhibitors of mammalian than of bacterial reductase [see, e.g., Burchall & Hitchings (1965)]. If trimethoprim analogues could be designed that, conversely, could adopt conformation LC2 but not conformation MA2, they should show a still greater selectivity than trimethoprim itself for binding to the bacterial enzyme.

Acknowledgments

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Registry No. Dihydrofolate reductase, 9002-03-3; trimethoprim, 738-70-5; 2,4-diaminopyrimidine, 156-81-0.

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Fatty Acid Acylation of Proteins in Bioluminescent Bacteria†

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ABSTRACT: Acylation of proteins with [^{3}H]tetradecanoic acid (+ATP) has been demonstrated in extracts of different strains of luminescent bacteria. The labeled polypeptides from *Photobacterium phosphoreum* (34K and 50K) have been identified as being involved in the acyl-protein synthetase activity that is part of a purified fatty acid reductase complex responsible for synthesis of long-chain aldehydes for the bioluminescent reaction. The two polypeptides (34K and 50K) have been separated from the acyl-CoA reductase enzyme (58K) of the complex and resolved from each other, and the 50K polypeptide was further purified to >95% homogeneity. Acylation of the 50K polypeptide, alone, occurred at a low rate; however, the rate and level of acylation were greatly stimulated by the addition of either the 34K or the 58K polypeptide. Cold chase experiments demonstrated that the acylated 50K polypeptide turned over in the presence of the 58K polypeptide but not in a mixture containing only the 34K and 50K polypeptides. Furthermore, the acylated 50K polypeptide could function as the immediate substrate for the fatty acyl-CoA reductase enzyme (58K), being reduced with NADPH to aldehyde. The 34K polypeptide was acylated only when all three polypeptides (34K, 50K, and 58K) were present. Fatty acid reductase activity could be restored by mixing of only the 58K (acyl-CoA reductase) and 50K polypeptides, showing that the 50K polypeptide is responsible for fatty acid activation in the fatty acid reductase complex and raising the question of what role the 34K polypeptide plays in fatty acid utilization in the luminescent system.

Studies concerning the incorporation of fatty acids into proteins have increased over the last few years. The formation of acyl-protein intermediates during fatty acid metabolism is well documented particularly with respect to the covalent attachment of fatty acids to acyl-carrier protein (ACP) as part of the mechanism of fatty acid synthesis (Vagelos, 1973; Rock et al., 1981; Jaworski & Stumpf, 1974). Studies on fatty acyl intermediates of other proteins involved in fatty acid metabolism are not extensive; however, evidence for this type of intermediate has been obtained in a few cases (Ayling et al., 1972; Bar-Tana et al., 1973). Interest in the acylation of proteins has also been stimulated by the recent discovery that fatty acids are covalently incorporated into specific membrane proteins as posttranslational events in both animal cells and viruses (Magee & Schlesinger, 1982; Schmidt & Schlesinger, 1979). Consequently, the isolation and study of enzymes responsible for acyl-protein formation are becoming of increasing biological importance.

In *Photobacterium phosphoreum*, the reduction of long-chain fatty acids to the corresponding aldehydes required for the luminescent reaction has recently been shown to involve the formation of acylated proteins (Riendeau et al., 1982). This activity, designated as acyl-protein synthetase, was measured by the incorporation of fatty acid into material insoluble in chloroform/methanol/acetic acid in an assay similar to that developed for measuring acyl-ACP synthetase activity (Ray & Cronan, 1976). The acyl-protein synthetase activity is part of a fatty acid reductase complex which can be resolved into acyl-protein synthetase and acyl-CoA reductase activities (Riendeau et al., 1982). Although the kinetics of formation of the acyl-protein were altered after

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