Finally, the amount of GDP associated with the eluted tubulin was taken to be equal to the expression

$$(\text{nmol of T–G})_{636} + \frac{2.00}{4.66}[(\text{nmol of G}_T)_{636} - (\text{nmol of T–G})_{636}]$$

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Nuclear Magnetic Resonance Studies of the Binding of Trimethoprim to Dihydrofolate Reductase†

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ABSTRACT: The resonances of the aromatic protons of trimethoprim [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine] in its complexes with dihydrofolate reductases from *Lactobacillus casei* and *Escherichia coli* cannot be directly observed. Their chemical shifts have been determined by transfer of saturation experiments and by difference spectroscopy using [2′,6′-$^2H_2$]trimethoprim. The complex of 2,4-diamino-5-(3′,4′-dimethoxy-5′-bromobenzyl)pyrimidine with the *L. casei* enzyme has also been examined. At room temperature, the 2′,6′-proton resonance of bound trimethoprim is very broad (line width >30 Hz); with the *E. coli* enzyme, the resonance sharpens with increasing temperature so as to be clearly visible by difference spectroscopy at 45 °C. This line broadening is attributed to an exchange contribution, arising from the slow rate of "flipping" about the C7–C1′ bond of bound trimethoprim. The transfer of saturation measurements were also used to determine the dissociation rate constants of the complexes. In the course of these experiments, a decrease in intensity of the resonance of the 2′,6′-proton resonance of free trimethoprim on irradiation at the resonance of the 6 proton of free trimethoprim was observed, which only occurred in the presence of the enzyme. This is interpreted as a nuclear Overhauser effect between two protons of the *bound* ligand transferred to those of the *free* ligand by the exchange of the ligand between the two states. The chemical shift changes observed on the binding of trimethoprim to dihydrofolate reductase are interpreted in terms of the ring-current shift contributions from the two aromatic rings of trimethoprim and from that of phenylalanine-30. On the basis of this analysis of the chemical shifts, a model for the structure of the enzyme–trimethoprim complex is proposed. This model is consistent with the (indirect) observation of a nuclear Overhauser effect between the 2′,6′ and 6 protons of bound trimethoprim.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The enzyme is of considerable pharmacological interest as the target for the "anti-folate" drugs. One of these, trimethoprim (I), is an effective antibacterial agent, selectively inhibiting bacterial dihydrofolate reductase by virtue of binding up to 50 000 times more tightly to the bacterial enzyme than it does to the mammalian enzyme (Hitchings & Burchall, 1965).

As part of a wider study aimed at understanding the factors determining inhibitor binding to dihydrofolate reductase, we have been using high-resolution NMR spectroscopy to study

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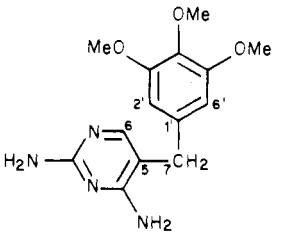


I

the inhibitor–enzyme complexes (Birdsall et al., 1977; Feeney et al., 1977; Roberts et al., 1977; Roberts, 1978). Among the most sensitive probes of the binding site of an inhibitor are the chemical shifts of the nuclei of the inhibitor itself. However, if the inhibitor binds so tightly that exchange between bound and free states is slow on the NMR time scale, it is frequently difficult to identify the ^1H resonances of the bound inhibitor because of overlap with the complex ^1H spectrum of the protein. In this paper, we describe the use of transfer of saturation experiments and difference spectroscopy to determine the chemical shifts of the ^1H resonances of trimethoprim in its complexes with the dihydrofolate reductases of *Lactobacillus casei* and *Escherichia coli*. These chemical shifts allow us to propose a model for the structure of the enzyme–trimethoprim complex.

Experimental Section

Materials. Dihydrofolate reductase was purified from *L. casei* MTX/R as described by Dann et al. (1976). The selectively deuterated enzyme was prepared by replacing the casein hydrolysate in the growth medium by a mixture of amino acids including deuterated histidine, phenylalanine and tryptophan and tyrosine deuterated at the 3′,5′ positions; for details, see Feeney et al. (1977). The trimethoprim-resistant strain *E. coli B* RT500 was kindly donated by Dr. J. J. Burchall (Burroughs Wellcome Research Laboratories, Research Triangle Park, NC). Dihydrofolate reductase was purified from this strain by a modification of the method of Dann et al. (1976). A column of DEAE-23 cellulose equilibrated in 25 mM Tris and 500 mM KCl at pH 8.5 was used to remove bound folate and related impurities (P. Scudder, R. W. King, and P. J. Cayley, unpublished experiments). The two constituent forms of the *E. coli* enzyme (Baccanari et al., 1977) were separated on an affinity column by using a modification of the method of D. Baccanari (personal communication). Both the *L. casei* and *E. coli* enzymes were lyophilized twice from ^2H_2O solution to remove exchangeable protons.

Trimethoprim (Sigma Chemicals Ltd.) was used without further purification. [2′,6′-$^2H_2$]Trimethoprim was prepared by dissolving 35 mg of trimethoprim in 0.25 mL of dimethyl sulfoxide, adding 20 mL of 1 M ^2HCl [CIBA (ARL) Ltd., Duxford, England] in ^2H_2O (Norsk Hydroelektrisk), and heating at 65 °C for 2.5 h. 2,4-Diamino-5-(3′,4′-dimethoxy-5′-bromobenzyl)pyrimidine was a generous gift from Dr. P. J. Goodford, Wellcome Research Laboratories, Beckenham, Kent, England.

NMR Spectroscopy. ^1H NMR spectra were obtained at 270 MHz in the Fourier transform mode by using a Bruker WH270 spectrometer equipped with a Nicolet 1180 computer. The samples had an enzyme concentration of ~1 mM in ^2H_2O containing 1 mM dioxane, 500 mM KCl, and 50 mM potassium phosphate, pH* 6.5 (*L. casei* enzyme) or pH* 6.8 (*E. coli* enzyme). The pH* values are meter readings uncorrected for the isotope effect on the glass electrode. Weighed amounts of solid trimethoprim were added; in the case of 2,4-diamino-5-(3′,4′-dimethoxy-5′-bromobenzyl)pyrimidine, they were first dissolved in 10 μL of [$^2H_6$]dimethyl sulfoxide.

Typically, 400 transients were averaged by using 4096 data points for a 4200-Hz spectral width (acquisition time of ~0.5 s). Before Fourier transformation, the free induction decay was multiplied by an exponential function, leading to a line broadening of 2 Hz, and a further 4096 zeroes added to give a final digital resolution of ~1 Hz/point. In the transfer of saturation experiments, selective irradiation was applied for up to 2 s immediately prior to the observing pulse (with a 1-ms interval to allow for electronic recovery), and a total pulse interval of 3 s was used. Chemical shifts are reported with respect to internal dioxane, whose resonance is 3.71 ppm downfield of 4,4-dimethyl-4-silapentanesulfonate.

Theory and Data Analysis. Determination of Dissociation Rate Constants. Forsén & Hoffman (1963) have described a number of double-resonance experiments which can be used to determine the lifetime of species undergoing slow exchange, and several workers [e.g., Redfield & Gupta (1971) and Campbell et al. (1977)] have adapted the original experiments to the Fourier transform mode of operation.

Consider a proton which exists in two magnetically distinct environments, for example, in a ligand free in solution and bound to a protein, and which exchanges sufficiently slowly between them for separate resonances to be observed for the two states. If the resonance of the bound state is saturated, this saturation will be transferred to the other state by means of the exchange process, and a decrease in the intensity of the resonance from the free state will result. The rate of decrease of the magnetization in the free state as a function of the time, t, for which the bound proton is saturated (assuming saturation occurs in a time short compared to t) is given by Forsén & Hoffman (1963) in eq 1, where M_{z0}^F is the equilibrium

$$\frac{dM_z^F}{dt} = \frac{M_{z0}^F}{T_{1F}} - \frac{M_z^F}{\tau_{1F}} \quad (1)$$

magnetization of the free proton in the absence of irradiation and τ_{1F} is the lifetime of the proton spin state defined by eq 2, where T_{1F} is the spin–lattice relaxation time and τ_F is the

$$\frac{1}{\tau_{1F}} = \frac{1}{T_{1F}} + \frac{1}{\tau_F} \quad (2)$$

residence time of the proton in the free state. The solution of eq 1 is given by eq 3. The free proton signal thus shows

$$M_z^F(t) = M_{z0}^F\left[\frac{\tau_{1F}}{\tau_F}\exp(-t/\tau_{1F}) + \frac{\tau_{1F}}{T_{1F}}\right] \quad (3)$$

an exponential decay, with a time constant τ_{1F}, to a new equilibrium value (eq 4). The value of τ_{1F} was determined

$$M_z^F(\infty) = M_{z0}^F\frac{\tau_{1F}}{T_{1F}} \quad (4)$$

by measuring the signal height as a function of t and fitting these data [in the form of a plot of $M_z^F(t) - M_z^F(\infty)$ vs. t] to eq 3 by nonlinear regression. Combination of this value with a measurement of $M_z^F(\infty)/M_{z0}^F$ (cf. eq 4) allows both τ_F and T_{1F} to be calculated. Then the dissociation rate constant can be calculated from eq 5, where P_F and P_B are the fractions

$$k_{off} = \frac{P_F}{P_B\tau_F} \quad (5)$$

of the total ligand in the free and bound states, respectively.

Calculation of Ring-Current Chemical Shifts. The Johnson–Bovey (1958) equation was used to calculate the

shielding effects of aromatic rings on the protons of bound trimethoprim in a wide range of different conformations; to calculate these shielding contributions it is necessary to know the coordinates of each proton with respect to the center of each aromatic ring. The trimethoprim molecule was positioned so that its pyrimidine ring coincided with the corresponding part of the pteridine ring of methotrexate in the *L. casei* enzyme–methotrexate–NADPH complex as determined by X-ray crystallography (Matthews et al., 1978) (atomic coordinates were generously provided by Dr. D. A. Matthews). The coordinates used for trimethoprim were taken from the neutron diffraction study of Koetzle & Williams (1976) or were calculated from standard bond lengths and angles; both sets gave essentially the same results. Ring-current shielding contributions were calculated as a function of the torsion angles θ_1 (C4–C5–C7–C1′) and θ_2 (C5–C7–C1′–C2′) by using 10° steps for each angle. In each of these 1296 conformations the shielding effects on the protons H6, H2′, and H6′ from each of the three aromatic rings (the pyrimidine and benzyl rings of trimethoprim and Phe-30) were calculated by transforming the coordinates so that each ring in turn was at the origin. For the diaminopyrimidine ring, the shielding effects calculated from the Johnson–Bovey equation were multiplied by a correction factor of 0.93 (Giessner-Prettre & Pullman, 1970; Giessner-Prettre et al., 1976).

Results

L. casei Dihydrofolate Reductase. The addition of 1 molar equiv of trimethoprim to *L. casei* dihydrofolate reductase led to extensive changes in the ^{1}H NMR spectrum, particularly in the aromatic region (Feeney et al., 1977). Because of the complexity of the spectrum, it is not surprising that the signals from bound trimethoprim could not be identified. Addition of a second equivalent of trimethoprim produced no further changes in the protein spectrum, but sharp signals from free trimethoprim were now clearly observed. This behavior is typical of that expected for a tightly binding ligand which is exchanging slowly between the bound and free states; in such a case separate signals would be expected for the nuclei in the two states. In an attempt to detect the aromatic signals of bound trimethoprim, we have examined the complex of trimethoprim with a selectively deuterated enzyme in which the only remaining resonances in the aromatic region are those of the 2,6 protons of the tyrosine residues. Figure 1a shows the aromatic region of the spectrum of the selectively deuterated enzyme in the presence of 2.7 molar equiv of trimethoprim. The 2,6-proton resonances of the five tyrosine residues and the two aromatic resonances of free trimethoprim are clearly observed. However, despite the simplicity of this region of the spectrum, it was still not possible to observe the aromatic resonances of bound trimethoprim. Furthermore, the complex formed by adding 1 equiv of [2′,6′-^{2}H$_2$]trimethoprim to the selectively deuterated enzyme gave a spectrum indistinguishable from that obtained by using the same concentration of nondeuterated trimethoprim. It is clear that the resonance of the 2′ and 6′ protons of bound trimethoprim must be very broad (line width greater than ~30 Hz) for it to be undetectable in these experiments.

The chemical shifts of the aromatic protons of bound trimethoprim were finally determined by means of transfer of saturation experiments. As can be seen from the spectrum in Figure 1a, for a sample containing 1 mM selectively deuterated dihydrofolate reductase and 2.7 equiv of trimethoprim the sharp H6 and H2′,H6′ resonances of free trimethoprim are clearly resolved from the tyrosine signals, making it easy to monitor their intensity in the presence of

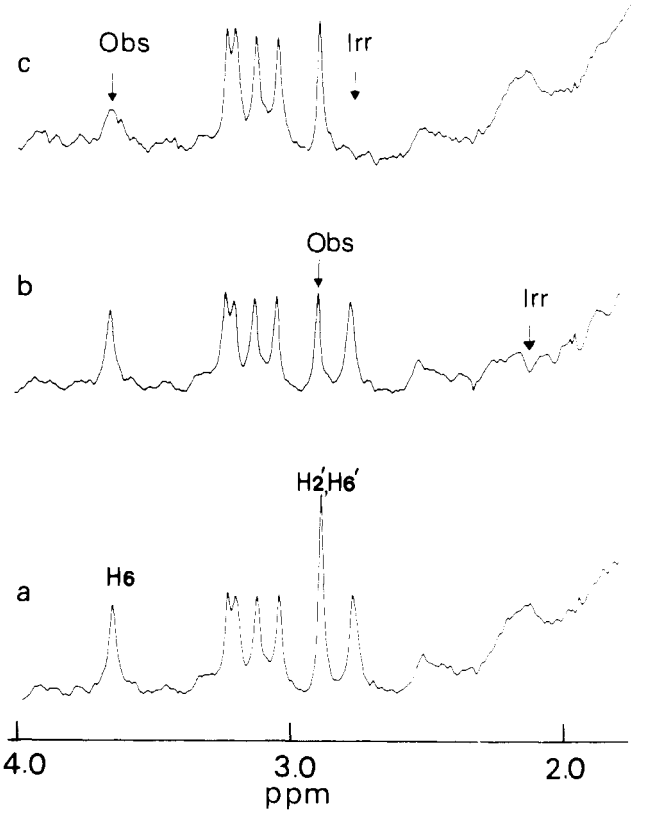


FIGURE 1: Aromatic region of the 270-MHz ^{1}H spectrum of selectively deuterated dihydrofolate reductase from *L. casei* in the presence of 2.7 molar equiv of trimethoprim at 45 °C. (a) Without irradiation, (b) with irradiation at 2.11 ppm, and (c) with irradiation at 2.76 ppm. The positions of irradiation and of those signals showing intensity changes are marked.

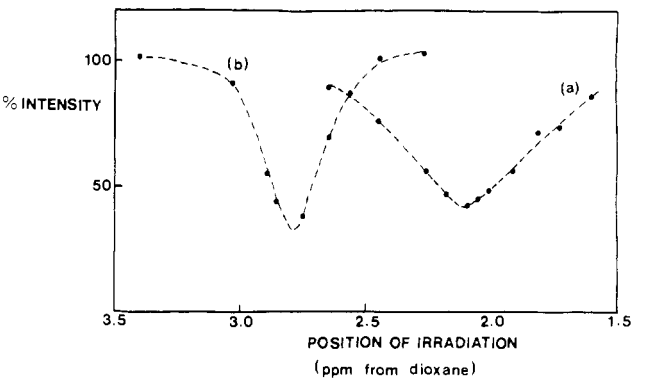


FIGURE 2: Variation in intensity of the resonances of (a) the 2′,6′ protons and (b) the 6 proton of free trimethoprim as a function of irradiation frequency in a solution containing 1 mM selectively deuterated dihydrofolate reductase from *L. casei* and 2.7 molar equiv of trimethoprim.

a second irradiating field. This irradiation, applied as a 0.4-s pulse, was located at 10-Hz intervals within a frequency range of 800 Hz on either side of the resonances of free trimethoprim. At temperatures below 30 °C no transfer of saturation was observed at any irradiation frequency, but at 40–45 °C transfer of saturation was observed.

Comparison of Figures 1a and 1b shows that a substantial decrease in the intensity of the H2′,H6′ resonance of free trimethoprim resulted when the irradiation was centered at 2.11 ppm from the dioxane reference at 40 °C. Similarly, a decrease in intensity of the free H6 signal was observed on irradiating at 2.76 ppm (compare Figures 1a and 1c). Figure 2 shows the variation of intensity of the two aromatic proton

Table I: ^{1}H Chemical Shifts of Trimethoprim and Related Compounds Free and Bound to the Dihydrofolate Reductases of *L. casei* and *E. coli*

compd	proton	chemical shift[a] (ppm) free[b]	*L. casei*	*E. coli* (form I)	*E. coli* (form II)
trimethoprim (I)	H6	3.62	2.76	2.61	2.79
	H2′,H6′	2.90	2.11	2.14	2.23
2,4-diamino-5-(3′,4′-dimethoxy-5′-bromobenzyl)pyrimidine (II)	H6	3.75	2.75		
	H2′,H6′	3.23, 3.34	2.70, 2.15		
2,4-diaminopyrimidine	H6	3.85	3.60[c]	3.67[d]	
2,4-diamino-5-methylpyrimidine (III)	H6	3.86[e]			
1-alkyl-3,4,5-trimethoxybenzene (IV)	H2,H6	2.71[e]			
1-alkyl-3,4-dimethoxy-5-bromobenzene (V)	H2,H6	3.01, 3.01[f]			

[a] In parts per million downfield of internal dioxane; estimated precision of bound chemical shifts is ±0.05 ppm. [b] Values quoted are for the protonated form of the pyrimidines. [c] From Feeney et al. (1977). [d] J. Cayley, unpublished experiments on a mixture of forms I and II. [e] S. Hurlbert, personal communication. [f] Estimated from substituent shift effects.

Table II: ^{1}H Chemical Shifts of Trimethoprim (I) and Its 5′-Bromo Analogue (II) in Complexes with Dihydrofolate Reductases

enzyme	ligand	bound chemical shift[a] (ppm) H6	H2′,H6′	4-OMe	3,5-OMe
E. coli (form I)	I	−1.26	−0.57	−0.04	−0.12
E. coli (form II)	I	−1.07	−0.48	−0.04	−0.16
L. casei	I	−1.10	−0.60	−0.03	≤0.11
	II	−1.11	−0.30, −0.85		

[a] Calculated by subtracting the chemical shift in the complex from that of the appropriate model compound, III, IV, or V (Table I). Negative shifts are upfield. For H6 and H2′,H6′ the errors are ±0.05 ppm and for 4-OMe and 3,5-OMe ±0.02 ppm.

resonances of free trimethoprim as a function of the frequency of irradiation. For each resonance there is an irradiation frequency which gives the maximum decrease in intensity, and this has been taken as the chemical shift of the corresponding proton of bound trimethoprim. The positions of the aromatic proton signals of bound trimethoprim are indicated in Figures 1b and 1c, and the chemical shifts of free and bound trimethoprim, together with those of some related compounds, are given in Tables I and II.

The H2′,H6′ resonance of bound trimethoprim is found to be 0.78 ppm to high field of the corresponding resonance of free trimethoprim, in a region of the spectrum where no signal is apparent, confirming the earlier indications that this resonance must be very broad. This would also explain the relatively broad range of irradiation frequencies which produced transfer of saturation effects on the H2′,H6′ resonance of free trimethoprim, almost twice the range effective for the H6 resonance. (It should be noted, however, that because it is necessary to produce saturation of the resonance of bound trimethoprim, the bandwidth of irradiation frequencies producing 50% or more of the maximum effect will be significantly greater than the true line width of the bound proton resonances [Gerig, 1977]).

For the H6 resonance of free trimethoprim, optimum transfer of saturation occurs on irradiation at a position 0.92 ppm to high field of the free trimethoprim signal, coincident with one of the tyrosine 2,6-proton resonances. This explains why the H6 resonance of bound trimethoprim was not detected in the earlier experiments; close examination of the spectrum in Figure 1 reveals that the resonance at 2.76 ppm does have a greater intensity than the other tyrosine 2,6-proton resonances. The position of the H6 resonance can be confirmed by the reverse transfer of saturation experiment. Figure 3

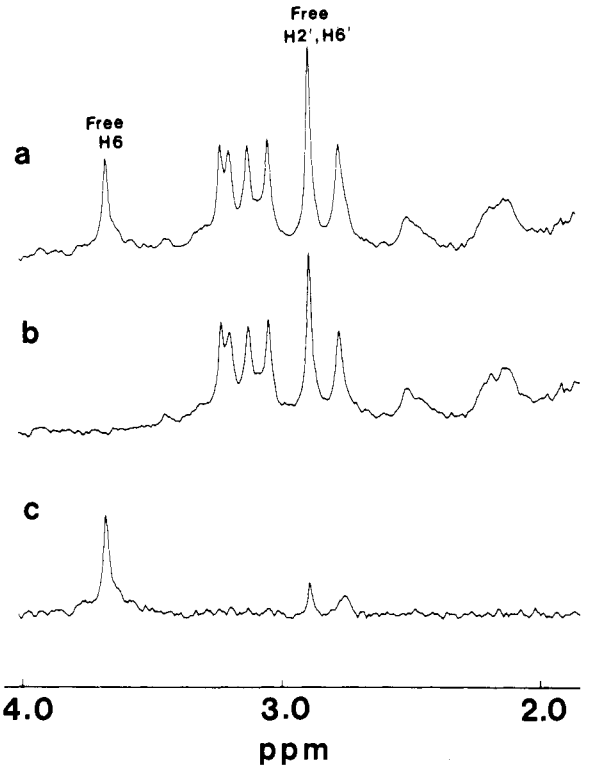


FIGURE 3: Aromatic region of the 270-MHz ^{1}H spectrum of selectively deuterated *L. casei* dihydrofolate reductase in the presence of 2.7 molar equiv of trimethoprim (a) without irradiation, (b) with irradiation at the resonance frequency of the 6 proton of free trimethoprim, and (c) the difference between parts a and b.

shows spectra obtained with irradiation at the position of the H6 resonance of free trimethoprim (Figure 3b) and with irradiation at a frequency well removed from these aromatic resonances (Figure 3a). The difference between these spectra (Figure 3c) shows clearly a relatively sharp signal at 2.76 ppm, corresponding to a decrease in intensity of the bound H6 resonance on saturation of the corresponding resonance of free trimethoprim. In addition, the difference spectrum shows a signal at the position of the H2′,H6′ resonance of free trimethoprim, which has decreased in intensity on irradiation of the H6 resonance. No such effect was observed in the absence of the enzyme. The simplest explanation of this observation is that H6 and H2′ (or H6′) are sufficiently close in space in *bound* trimethoprim for there to be a negative nuclear Overhauser effect on the H2′,H6′ signal on saturation of the H6 resonance. This effect is then transmitted to the *free* species by the exchange of trimethoprim molecules between the two

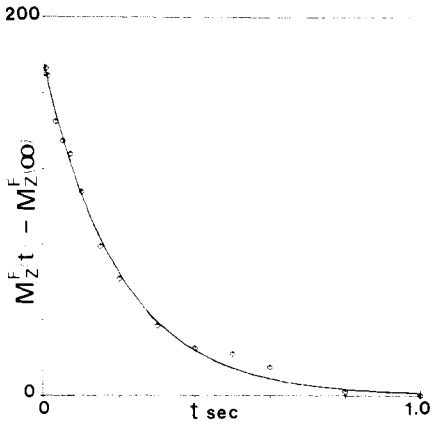


FIGURE 4: Change in magnetization, $M_z^F(t) - M_z^F(\infty)$, of the H6 resonance of free trimethoprim as a function of the time, t, for which the bound resonance was irradiated. The curve is the best least-squares fit to the data, calculated by using the parameters in Table III.

Table III: Dissociation Rate Constants, Spin Lifetimes, and Relaxation Times in the Complexes of Trimethoprim with Dihydrofolate Reductases at 45 °C

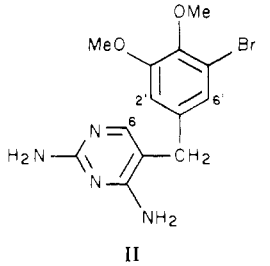
enzyme	observed signal	τ_{1F} (s)	T_{1F} (s)	k_{off} (s^{-1})
L. casei	H2′,H6′	0.22	0.72	5.2
	H6	0.20	1.22	6.8
E. coli (form II)	H2′,H6′	0.12	0.15	6.7

states in a time comparable to or less than the spin–lattice relaxation times. This novel "transferred" nuclear Overhauser effect is discussed in more detail elsewhere (Albrand et al., 1979).

We have also measured the chemical shifts of some of the nonaromatic protons of trimethoprim in its 1:1 complex with dihydrofolate reductase; small upfield shifts (0.03–0.11 ppm) of the methoxy protons were observed on binding, but the signals of the CH_2 group could not be detected.

As indicated by eq 3–5 above, it is possible to use the transfer of saturation experiment to determine the dissociation rate constant of the complex by measuring the intensity of a resonance of free trimethoprim as a function of the length of time for which the corresponding bound resonance is irradiated. Figure 4 shows the results of such experiments for the H6 resonance of trimethoprim. The line is the "best-fit" exponential, determined by nonlinear regression; the parameters derived in this way and the dissociation rate constant calculated from them are given in Table III. Similar results are obtained from the H2′,H6′ resonances.

The complex of *L. casei* dihydrofolate reductase with 2,4-diamino-5-(3′,4′-dimethoxy-5′-bromobenzyl)pyrimidine (II), an analogue of trimethoprim having an unsymmetrically substituted benzyl ring, has also been examined.



II

Using a solution containing a molar ratio of ligand/enzyme of 2:1, we have determined the chemical shifts of the ligand protons in the complex by transfer of saturation experiments at 45 °C. The resonance of the H6 proton of the bound ligand

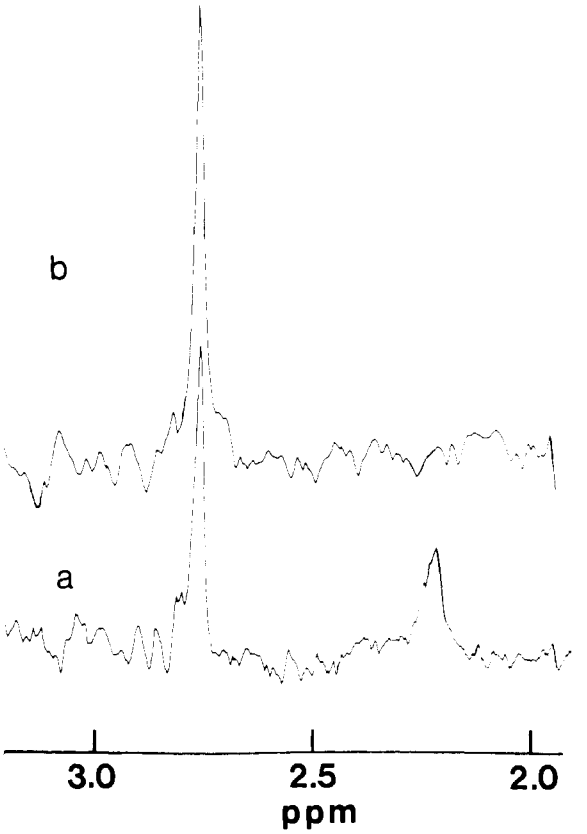


FIGURE 5: Difference spectra obtained by subtracting the spectrum of the complex of *E. coli* dihydrofolate reductase with [2′,6′-$^2H_2$]-trimethoprim from that of the corresponding complex with normal trimethoprim. Sample temperature: (a) 45 °C and (b) 10 °C.

was found to be centered at 2.75 ppm, and those of the two protons on the benzyl ring (H2′ and H6′) were at 2.70 and 2.15 ppm (see Tables I and II). Unfortunately, the resonances of H2′ and H6′ in II have very nearly the same chemical shift in the free ligand and cannot be individually assigned in either the free or the bound state.

E. coli Dihydrofolate Reductase. We have studied the complexes of trimethoprim with each of the two forms of the enzyme (Baccanari et al., 1977) and also with the mixture. The two forms bind trimethoprim with substantially different equilibrium constants [K_d (form I) $< 10^{-8}$ M; K_d (form II) $= (1.0 \pm 0.2) \times 10^{-7}$ M determined from fluorescence quenching of the enzyme], and it was hoped that the ^1H spectra of the complexes might reflect this difference.

For each form of the enzyme the addition of 1 equiv of trimethoprim produces extensive changes throughout the whole spectrum. As in the case of the complex with the *L. casei* enzyme, it was not possible to distinguish the bound trimethoprim proton signals from those of the enzyme. Addition of a further equivalent of trimethoprim gave rise to signals at the frequency positions of those in free trimethoprim, as would be expected for a tightly binding ligand. We have located some of the bound trimethoprim proton signals both by the transfer of saturation method and in the difference spectra from the complexes formed with deuterated and normal trimethoprim.

If the spectrum obtained from a solution containing *E. coli* dihydrofolate reductase (mixture of forms I and II) and 2 molar equiv of [2′,6′-$^2H_2$]trimethoprim is subtracted from that obtained from a solution of identical composition except that it contains normal rather than deuterated trimethoprim, the resulting difference spectrum should contain only the resonances of the 2′,6′ protons in the bound and free states. At

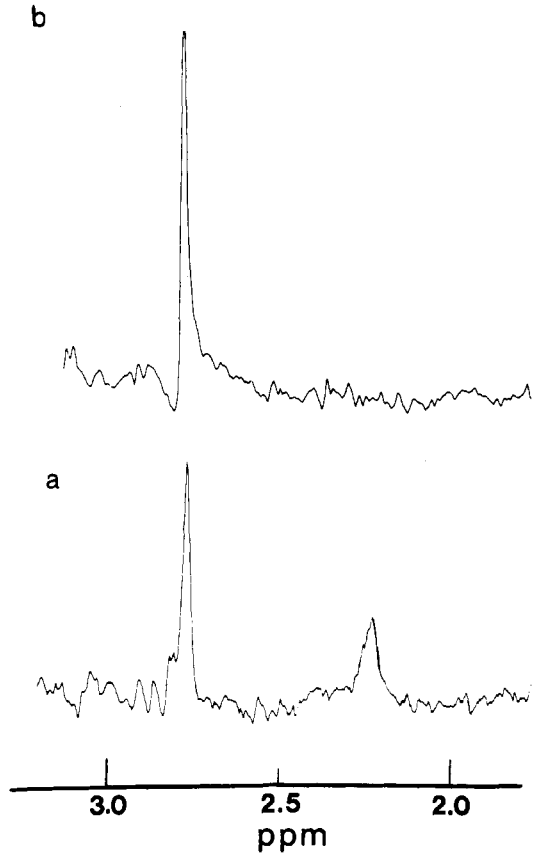


FIGURE 6: Difference spectra obtained by subtracting the spectrum of a complex of *E. coli* dihydrofolate reductase with [2′,6′-$^2H_2$]-trimethoprim from that of the corresponding complex with normal trimethoprim with a sample temperature of 45 °C. (a) Binary enzyme–trimethoprim complexes; (b) ternary enzyme–NADPH–trimethoprim complexes.

10 °C, the difference spectrum showed only a signal corresponding to the free ligand (Figure 5b). When the temperature was raised to 45 °C, an additional relatively sharp signal (line width 14 Hz) from the 2′,6′ protons of the bound ligand was observed at 2.10 ppm (Figure 5a). The line width remained unchanged when the temperature was further increased to 55 °C. When the coenzyme $NADP^+$ was added to the complexes at 45 °C there was no change in the line width of the bound H2′,H6′ signal in the difference spectrum, but the addition of NADPH apparently caused substantial broadening such that the bound H2′,H6′ signal could no longer be detected (Figure 6).

The assignment of the peak at 2.10 ppm to bound H2′,H6′ protons has been confirmed by transfer of saturation experiments on complexes with the two separate forms of the enzyme. Transfer of saturation was detected at temperatures above 30 °C for complexes with form II of the enzyme and above 40 °C for complexes with form I.

The results are summarized in Tables I and II; the 6 proton of trimethoprim has a somewhat different shift in complexes with the two forms of the enzyme (a shift difference of 0.18 ± 0.05 ppm). An indication of this had been noted in earlier transfer of saturation experiments on the mixture of forms I and II, in which a double minimum was found in plots analogous to Figure 2 for the H6 resonance. The shift difference between the H2′,H6′ signals in the two complexes is much smaller (0.09 ± 0.05 ppm) and was not detected in the difference spectrum of Figure 5. On addition of $NADP^+$ to form the ternary complex, the resonance of H6 of trimethoprim bound to form II of the enzyme did not change, but the corresponding signal from the complex with form I shifted upfield so that the shift difference between the two forms is abolished.

Irradiation of the resonance of H2′,H6′ of bound trimethoprim caused, in addition to the large decrease in the intensity of the signal from free H2′,H6′, a small decrease in the intensity of the H6 signal of free trimethoprim, and a similar small decrease of the free H2′,H6′ signal on irradiation of the bound H6 resonance was also observed. These effects are similar to those described above for the *L. casei* enzyme and also arise from the "transferred" nuclear Overhauser effect, implying the existence of a nuclear Overhauser effect between H6 and H2′ or H6′ of bound trimethoprim. The relatively poor signal-to-noise ratio in the difference spectrum prevented us from confirming this directly by observation of the H2′,H6′ signal of bound trimethoprim.

It is more difficult to detect the resonances from the methylene and methoxy protons of bound trimethoprim. One methoxy signal could be identified in both form I and form II in the difference spectrum between free enzyme and the complex formed by adding 0.9 molar equiv of trimethoprim. This was shown by transfer of saturation experiments to arise from the two equivalent 3′,5′-methoxy groups. The changes in chemical shifts on binding are 0.12 (form I) and 0.16 ppm (form II) upfield. Another sharp signal in the difference spectrum probably arises from the 4-methoxy group, with an upfield shift of 0.04 ppm on binding to either form I or form II. It is not possible to exclude the possibility that this signal comes from the methylene protons, although one would expect these to be nonequivalent in bound trimethoprim and thus to give rise to a quartet.

The dissociation rate constant of trimethoprim from form II of the *E. coli* enzyme has been determined by the transfer of saturation method as outlined above for the *L. casei* enzyme, and the results are given in Table III. The value of k_{off} obtained is in good agreement with that of 8.50 (±0.05) s^{-1} obtained by stopped-flow measurements at lower temperatures (R. W. King and J. Cayley, unpublished experiments). For form I, only a small decrease in intensity of the free ligand resonances was observed in the transfer of saturation experiment, and a reliable measurement of the dissociation rate constant was not possible.

Discussion

It is clear from the chemical shifts summarized in Tables I and II that the environment of trimethoprim is broadly similar when bound to any of the three dihydrofolate reductases studied here. The differences, for example, between forms I and II of the *E. coli* enzyme, are significant but small. In particular, the characteristics of the H2′,H6′ resonance of bound trimethoprim are the same in each case. Only a single resonance is detected for these two protons, implying that they are in an identical environment, although one would expect some difference in the environment on opposite sides of the benzyl ring of bound trimethoprim in at least one of the enzymes. Furthermore, this resonance is too broad to be observed directly at room temperature, although it does sharpen sufficiently to be observable for the *E. coli* enzymes at 45 °C. A simple exchange of trimethoprim between the bound and free states cannot be responsible for this selective broadening since no broadening of the free H2′,H6′ resonance is seen. These two characteristics of the H2′,H6′ resonance can only be explained if in bound trimethoprim there is relatively rapid "flipping" of the benzyl ring about the methylene–C1′ bond (that is, interconversion between two chemically indistinguishable conformations related by a 180°

rotation about this bond). This would exchange the positions of the 2′ and 6′ protons and, if sufficiently rapid, would lead to an averaging of their chemical shifts into a single resonance line. If the rate of "flipping" were not sufficient for complete averaging of the two environments, a very broad line, corresponding to so-called intermediate exchange, would be obtained. Thus, with the *E. coli* enzyme, the rate of "flipping" about the methylene–C1′ bond at 10 °C is such that the H2′,H6′ signal is so broadened by exchange as to be unobservable. However, the increase in the rate of "flipping" produced by raising the temperature to 45 °C is sufficient to lead to complete averaging of the two environments on the NMR time scale and thus to a sharp observable signal.

Such a rapid "flipping" of a symmetrically substituted aromatic ring in a bound ligand is clearly analogous to the "flipping" of the aromatic rings of tyrosine residues, which is now well established for a number of proteins (Snyder et al., 1975; Wagner et al., 1975; Campbell et al., 1975; Feeney et al., 1977). For example, the observation that the five [3′,5′-$^2H_2$]tyrosine residues in selectively deuterated dihydrofolate reductase give rise to only five aromatic proton resonances (see Figure 1) shows that here the "flipping" about the C_β–C_γ bond is sufficiently fast in each tyrosine to average the environments of the 2′ and 6′ protons completely.

The symmetry of the benzyl ring in trimethoprim requires that the two conformations related by the 180° "flip" be equally populated, so the observed shift change is simply the average of those characteristic of the two sides of the ring. This is not necessarily true, however, of the unsymmetrical 5′-bromo analogue II. Here again, only a single set of resonances was detected for the bound ligand, implying either a single fixed conformation about the methylene–C1′ bond or *rapid* interconversion between two or more conformations. In view of the not too dissimilar size of the bromo and methoxy groups and the closely similar chemical shift of H6 in trimethoprim and its 5′-bromo analogue, it seems reasonable to assume that they bind to the enzyme in essentially the same way, the only difference being in the relative population of the two conformations about the methylene–C1′ bond. If the 5′-bromo compound adopts one of these two conformations *exclusively*, then the observed shifts, 0.30 and 0.85 ppm, are those characteristic of the environments on the two sides of the aromatic ring. The average of these two is 0.575 ppm, close to the observed value of 0.6 ppm for trimethoprim. Since any averaging between the two conformations will reduce the difference between the two observed shifts in the 5′-bromo compound, the *minimum* shift difference between the two sides of the benzyl ring must be 0.6 ppm. Averaging of these two resonances will occur if the rate of "flipping" is greater than 2π times their chemical shift difference, so that the *minimum* rate of "flipping" of the benzyl ring of trimethoprim bound to the *E. coli* enzyme is 10^3 s^{-1} at 45 °C.

Thus, the 6 proton and one of the 2′,6′ protons of trimethoprim experience a relatively large upfield shift on binding to dihydrofolate reductase, while the other aromatic proton and the methoxy protons undergo much smaller chemical shift changes. In general it is difficult to give precise structural interpretations of chemical shift changes observed for ligand nuclei on binding to an enzyme because of the multiplicity of possible contributions to such changes. For trimethoprim, one contribution which can be clearly identified is the ring-current effect of each of the aromatic rings of trimethoprim on the chemical shifts of the protons of the other ring. This will depend strongly on the conformation of the molecule. In solution, free trimethoprim exists as a mixture of many rapidly interconverting conformations about the C5–methylene (torsion angle θ_1) and methylene–C1′ (angle θ_2) bonds (Koetzle & Williams, 1976), so the observed chemical shifts will contain an averaged contribution from this source. On binding to the enzyme, the molecule adopts a single conformation, resulting in a change in these "internal" ring-current shifts. Some indication that this makes a contribution to the observed chemical shift changes comes from a comparison with 2,4-diaminopyrimidine. This has no conformation-dependent "internal" ring-current shift contribution and seems to bind to the enzyme in the same way as the corresponding ring of trimethoprim, as judged by the very similar effects of both compounds on the tyrosine and histidine residues of the enzyme (Birdsall et al., 1977; Feeney et al., 1977; Roberts et al., 1977). The 6 proton of 2,4-diaminopyrimidine experiences as upfield shift of only 0.18–0.25 ppm, compared to 0.83–1.01 ppm for H6 of trimethoprim (Table I), suggesting a substantial contribution from the "internal" ring-current shift. Trimethoprim itself is clearly not a suitable reference compound for comparison with the chemical shifts in the complex since its chemical shifts in the free state already have contributions from these "internal" shifts. For this reason we have expressed the bound shifts in Table II relative to those of the model compounds III and IV, which have no contributions from conformation-dependent "internal" ring-current shifts. The "true" bound shifts of the 6 proton of trimethoprim is then 1.10–1.26 ppm upfield; if ~0.2 ppm of this comes from the same (as yet unknown) source as the H6 bound shift in 2,4-diaminopyrimidine, we can calculate which conformations of bound trimethoprim would give a large enough "internal" contribution to account for the total shift. Acceptable agreement is found for a fairly broad range of conformations in the range $\theta_1 = 190 \pm 50°$, $\theta_2 = 90 \pm 50°$ (because of the symmetry about the methylene–C1′ bond, there is an equivalent group of solutions with $\theta_2 = 270 \pm 50°$; these ranges are more restrictive than they appear since there is a marked correlation between θ_1 and θ_2 for acceptable conformations).

However, none of these conformations gave acceptable agreement with the observed (averaged) H2′,H6′ chemical shift when only the "internal" ring-current shift contributions were considered; it proved impossible to obtain large enough upfield shifts for all of the protons simultaneously. Thus, it appears that, although the "internal" shifts make a contribution to the observed bound shifts, an additional source(s) of shielding must also be considered. To progress further, we must use the crystallographically determined structure of the *L. casei* enzyme–methotrexate–NADPH complex (Matthews et al., 1978) to identify neighboring amino acid residues which might contribute to the chemical shift of the trimethoprim protons. This involves the assumption that the 2,4-diaminopyrimidine ring of trimethoprim binds in the same position as the corresponding part of the pteridine ring of methotrexate and that there is no significant difference in protein conformation between the two complexes. What evidence there is, principally from NMR spectroscopy, is consistent with these assumptions. Trimethoprim and methotrexate produce similar effects on the histidine resonances of the *L. casei* enzyme (Birdsall et al., 1977; Roberts et al., 1977) with the exception of one resonance which probably arises from a histidine interacting directly with the glutamate moiety of methotrexate (Birdsall et al., 1977; B. Birdsall, A. Gronenborn, J. Feeney, and G. C. K. Roberts, unpublished experiments). Their effects on the tyrosine and on the high-field methyl resonances are also very similar (Feeney et al., 1977; Roberts et al., 1977; B. Birdsall, B. J. Kimber, J.

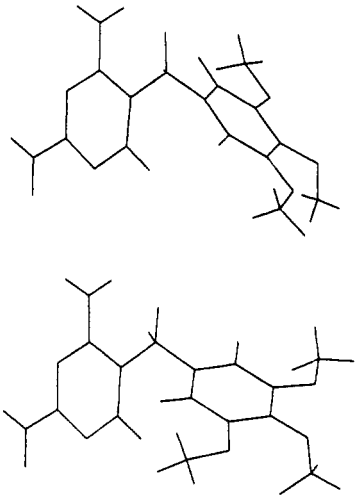


FIGURE 7: Two possible conformations of trimethoprim in its complex with dihydrofolate reductase, as calculated from the chemical shift changes on binding. The upper molecule has $\theta_1 = 205°$ and $\theta_2 = 100°$, and the lower has $\theta_1 = 190°$ and $\theta_2 = 45°$. These two torsion angles are defined by the atoms C4–C5–C7–C1′ (θ_1) and C5–C7–C1′–C2′ (θ_2); the torsion angles are zero when atoms α–β–γ–δ are synplanar, and a positive rotation is one which moves atom δ in a clockwise sense when looking along the β–γ bond from β to γ.

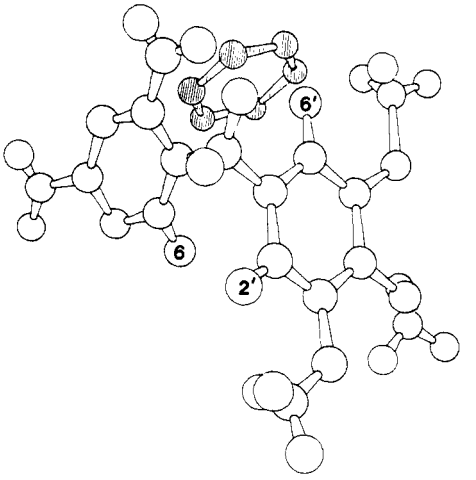


FIGURE 8: One of the two possible conformations of trimethoprim in its complex with dihydrofolate reductase, showing its relationship to the aromatic ring of Phe-30 (shaded). This conformation has $\theta_1 = 205°$ and $\theta_2 = 100°$.

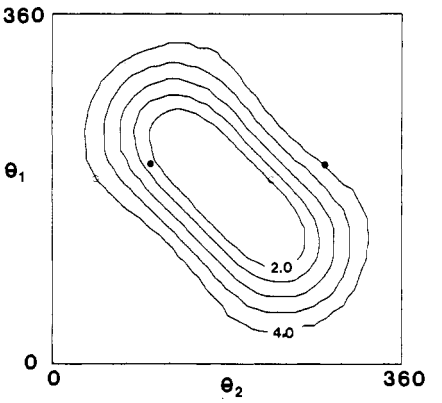


FIGURE 9: Distance between H6 and H2′ of trimethoprim calculated as a function of the torsion angles θ_1 and θ_2. The contours are at 2.0, 2.5, 3.0, 3.5, and 4.0 Å. Because of the symmetry of the trimethoxybenzyl ring, the diagram also shows the H6–H6′ distance if a 180° "phase shift" is introduced on the θ_2 axis. The points are the positions of the 2′ and 6′ protons in the two calculated conformations: (●) $\theta_1 = 205°$, $\theta_2 = 100°$; (○) $\theta_1 = 190°$, $\theta_2 = 45°$.

Feeney, and G. C. K. Roberts, unpublished experiments), only one residue showing small differences in each case. The protonated forms of both molecules bind to the enzyme more strongly than the neutral species (Hood & Roberts, 1978); the change in pK on binding is larger for methotrexate than for trimethoprim, but this does not necessarily imply a difference in the mode of binding of the protonated form. The presence of coenzyme also seems to have minor effects, since it leads to only small changes in the chemical shifts of bound trimethoprim, while trimethoprim itself produces very much the same changes in the histidine and tyrosine resonances in the presence or absence of NADPH (Birdsall et al., 1977; Feeney et al., 1977; Roberts et al., 1977; B. Birdsall, A. Gronenborn, J. Feeney, and G. C. K. Roberts, unpublished experiments).

Examination of the crystal structure of the enzyme–methotrexate–NADPH complex (Matthews et al., 1978) shows that there are three residues near the pteridine ring of methotrexate which might affect the chemical shifts of the proton resonances of bound trimethoprim: Asp-26, Phe-30, and Phe-49. If the pyrimidine ring is bound in the same place as the corresponding part of methotrexate, the distance and orientation of the C6–H6 bond with respect to the carboxylate group of Asp-26 would be such that the electric field shift (Buckingham, 1960; Batchelor, 1975; Batchelor et al., 1975) of the H6 resonance would be expected to be small and *downfield*; any contribution to the observed shift is clearly not dominant. The ring-current shielding contributions from Phe-49 would also be expected to be small since the center of the ring is 7.4 Å from H6. Furthermore, this residue is not conserved in the enzyme from *E. coli* (Stone et al., 1977), and yet the chemical shifts of all of the trimethoprim proton resonances are very similar in the complexes with the *L. casei* and *E. coli* enzymes, indicating that contributions from Phe-49 are negligible. In contrast, the aromatic ring of Phe-30, which is present in both enzymes, is much closer to trimethoprim in its assumed binding position and is the obvious candidate for the source of the additional shielding contribution. We have therefore calculated the chemical shifts of the H6, H2′, and H6′ resonances of trimethoprim as a function of the dihedral angles θ_1 and θ_2, taking into account the ring-current contributions from the pyrimidine and benzyl rings of trimethoprim and that of Phe-30, the position of Phe-30 relative to the pyrimidine ring being maintained precisely the same as that observed in the crystal of the methotrexate complex (Matthews et al., 1978). Initially no account was taken of van der Waals contacts, the sole criterion being agreement of the calculated shifts with those observed with the *L. casei* enzyme (H6, −1.10 ppm; H2′,H6′ mean, −0.60 ppm; difference, ≥0.6 ppm). Two solutions were obtained, both well-defined. With $\theta_1 = 190°$ (±10°) and $\theta_2 = 45°$ (±5°), we calculate the bound shift of H6 as −1.03 ppm and the mean of the shifts of H2′ and H6′ as −0.64 ppm. The second solution, $\theta_1 = 205°$ (±10°) and $\theta_2 = 100°$ (±10°), gives an H6 shift of −1.10 ppm and a mean shift of H2′ and H6′ of −0.58 ppm. (Again there are indistinguishable solutions at θ_2 +180°.) Both of these solutions give calculated shifts in excellent agreement with those observed, and we cannot choose between them on chemical shift grounds; they are illustrated in Figures 7 and 8.

An independent test of these proposed conformations is available from the (indirect) observation of a nuclear Overhauser effect between the 6 and 2′ (or 6′) protons of bound trimethoprim, which implies that these protons are in close proximity. It can be calculated (Albrand et al., 1979) that a significant nuclear Overhauser effect would be observed in experiments such as that shown in Figure 3 only if the two

Table IV: Calculated Contributions to the Chemical Shift Changes in Trimethoprim on Binding to *L. casei* Dihydrofolate Reductase

proton	chemical shift contribution[a] (ppm)		
	internal[b]	Phe-30	total
H6	−0.88	−0.22	−1.10
H2′	0.29	−0.14	0.15
H6′	0.03	−1.33	−1.30

[a] Positive shifts are downfield. [b] For H6, from the trimethoxybenzyl ring; for H2′ and H6′, from the pyrimidine ring.

protons involved are within 4 Å of one another. Figure 9 shows the distance between H6 and H2′ (or H6′) of trimethoprim as a function of the torsion angles θ_1 and θ_2. It can be seen that the nuclear Overhauser effect experiment places a significant restriction on the region of conformational space which could be occupied by bound trimethoprim and that both the conformations calculated from the chemical shift data fall comfortably within this region. In fact no distinction between the two solutions is possible by this experiment either since in both conformations one of the benzyl ring protons is essentially in van der Waals contact with H6 (2.1–2.3 Å) while the other is some 4 Å away.

The two possible conformations are in fact quite similar, as shown in Figure 7. The value of θ_1 is very similar in both of them, differing by some 70° from that observed for free trimethoprim in the crystal (Koetzle & Williams, 1976). This has the effect of bringing the benzyl ring to lie "beside" the pyrimidine ring, and in this conformation trimethoprim occupies, very roughly, the same space as the pteridine and aminobenzoyl rings of methotrexate. The angle between the two rings of trimethoprim is largely governed by θ_2 and differs by 55° between the two conformations. It can be seen from Figure 7 that the benzyl ring proton (either H2′ or H6′) which is closest to H6 is either above or below the plane of the pyrimidine ring, depending on the value of θ_2.

The various contributions to the bound shifts of trimethoprim in the conformation having $\theta_1 = 205°$ and $\theta_2 = 100°$ are enumerated in Table IV. As was indicated by the observations with the 5′-bromo analogue II, the two protons on the trimethoxybenzyl ring experience very different shifts, by far the largest shift contribution coming from Phe-30. In contrast (as argued above), the bulk of the bound shift of H6 is an "internal" ring-current shift from the benzyl ring of trimethoprim. The calculated contribution to the shift of H6 from Phe-30 (−0.22 ppm) is very similar to that observed with 2,4-diaminopyrimidine (−0.25 ppm); this is entirely consistent with our hypothesis that the pyrimidine ring of diaminopyrimidine (and trimethoprim) binds in the same way as the corresponding part of methotrexate. The slightly different chemical shift changes observed for binding to the two forms of the *E. coli* enzyme can be readily accounted for on the present model by changes of about 5° in θ_1 and/or θ_2 from the values which best fit the *L. casei* shifts.

The proposed conformation depends critically on our assumption that the pyrimidine ring binds in just the same way as the corresponding part of the pteridine ring of methotrexate (and on our having identified all of the significant chemical shift contributions). The evidence supports this mode of binding, but since only a limited number of residues of the protein have been studied a conformational change which would affect the proposed model and yet go undetected cannot be ruled out. If by examining additional resolved resonances from the protein it can be established that the protein structure does remain largely invariant in the two complexes, then the approach described here could be of general usefulness in studies of ligand binding to proteins. By using crystallographic information on one complex to help establish the origin of chemical shift changes and NMR signals from the protein to monitor the protein structure, one can obtain detailed structural information about related complexes which have not been, or perhaps cannot be, examined crystallographically.

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Mechanism of Actomyosin Adenosine Triphosphatase. Evidence That Adenosine 5′-Triphosphate Hydrolysis Can Occur without Dissociation of the Actomyosin Complex†

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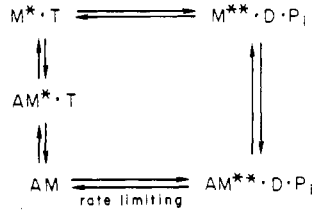
ABSTRACT: We have investigated the steps in the actomyosin ATPase cycle that determine the maximum ATPase rate (V_{max}) and the binding between myosin subfragment one (S-1) and actin which occurs when the ATPase activity is close to V_{max}. We find that the forward rate constant of the initial ATP hydrolysis (initial P_i burst) is about 5 times faster than the maximum turnover rate of the actin S-1 ATPase. Thus, another step in the cycle must be considerably slower than the forward rate of the initial P_i burst. If this slower step occurs only when S-1 is complexed with actin, as originally predicted by the Lymn–Taylor model, the ATPase activity and the fraction of S-1 bound to actin in the steady state should increase almost in parallel as the actin concentration is increased. As measured by turbidity determined in the stopped-flow apparatus, the fraction of S-1 bound to actin, like the ATPase activity, shows a hyperbolic dependence on actin concentration, approaching 100% asymptotically. However, the actin concentration required so that 50% of the S-1 is bound to actin is about 4 times greater than the actin concentration required for half-maximal ATPase activity. Thus, as previously found at 0 °C, at 15 °C much of the S-1 is dissociated from actin when the ATPase is close to V_{max}, showing that a slow first-order transition which follows the initial P_i burst (the transition from the refractory to the nonrefractory state) must be the slowest step in the ATPase cycle. Stopped-flow studies also reveal that the steady-state turbidity level is reached almost instantaneously after the S-1, actin, and ATP are mixed, regardless of the order of mixing. Thus, the binding between S-1 and actin which is observed in the steady state is due to a rapid equilibrium between S-1–ATP and acto–S-1–ATP which is shifted toward acto–S-1–ATP at high actin concentration. Furthermore, both S-1–ATP and S-1–ADP·P_i (the state occurring immediately after the initial P_i burst) appear to have the same binding constant to actin. Thus, at high actin concentration both S-1–ATP and S-1–ADP·P_i are in rapid equilibrium with their respective actin complexes. Although at very high actin concentration almost complete binding of S-1–ATP and S-1–ADP·P_i to actin occurs, there is no inhibition of the ATPase activity at high actin concentration. This strongly suggests that both the initial P_i burst and the slow rate-limiting transition which follows (the transition from the refractory to the nonrefractory state) occur at about the same rates whether the S-1 is bound to or dissociated from actin. We, therefore, conclude that S-1 does not have to dissociate from actin each time an ATP molecule is hydrolyzed.

It is now generally accepted that muscle contraction is caused by interdigitating myosin and actin filaments sliding past each other. This sliding process appears to be driven by a cyclic interaction of myosin cross bridges with actin and ATP (Huxley, 1969; Huxley, 1974). For an understanding of this cyclic interaction in vivo, considerable effort has been devoted to studying the kinetics of the actomyosin ATPase in vitro. The major goal of these studies, most of which have employed the soluble fragments of myosin, heavy meromyosin (HMM), and subfragment 1 (S-1), has been to determine the simplest kinetic model which is consistent with both pre-steady-state and steady-state kinetic data.

In 1971, on the basis of their pre-steady-state kinetic studies, Lymn & Taylor (1971) proposed the model shown in Scheme I for the actomyosin ATPase. In this model the number of asterisks on the myosin intermediates qualitatively represents the amount of fluorescence shown by these intermediates (Bagshaw et al., 1974). This model was based on data obtained at 20 °C which showed that, at relatively low actin concentration, complete dissociation of the acto–HMM complex by ATP occurs before hydrolysis of the ATP in the initial P_i burst ($M^*{\cdot}T \rightarrow M^{**}{\cdot}D{\cdot}P_i$). Sleep & Taylor (1976), working at low temperature, confirmed this basic finding. However, it has never been clear what rate constants are required in this model to explain the steady-state data of Eisenberg (Eisenberg & Moos (1968, 1970)). Since the initial P_i burst was found to be faster than the steady-state maximum

Scheme I



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