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Dynamics of trimethoprim bound to dihydrofolate reductase

(¹H NMR/¹³C NMR/nuclear magnetic relaxation/fluctuations/hydrogen bonds)

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ABSTRACT **The conformation of a small molecule in its binding site on a protein is a major factor in the specificity of the interaction between them. In this paper, we report the use of ¹H and ¹³C NMR spectroscopy to study the fluctuations in conformation of the anti-bacterial drug trimethoprim when it is bound to its "target," dihydrofolate reductase. ¹³C relaxation measurements reveal dihedral angle changes of ±25° to ±35° on the subnanosecond time scale, while ¹³C line-shape analysis demonstrates dihedral angle changes of at least ±65° on the millisecond time scale. ¹H NMR shows that a specific hydrogen bond between the inhibitor and enzyme, which is believed to make an important contribution to binding, makes and breaks rapidly at room temperature.**

A knowledge of the internal motions of proteins is of considerable importance for understanding their structure–function relationships (1–4). In recent years, a variety of theoretical (4–6) and experimental (3, 7–14) methods have been brought to bear on this problem, and a picture of the kinds of motion that take place is beginning to emerge. Hitherto, most attention has been focused on the atoms of the protein itself, although similar dynamic behavior would be expected for small molecules bound to proteins, and this has indeed been observed in a few cases (10, 15–18). We have been studying the binding of the antibacterial drug trimethoprim (Fig. 1) to its "target" dihydrofolate reductase, and we have now used ¹H and ¹³C NMR line-shape and relaxation measurements to study the conformational dynamics of the drug molecule in its complex with *Lactobacillus casei* dihydrofolate reductase.

MATERIALS AND METHODS

Dihydrofolate reductase from *L. casei* was purified as described (19). The synthesis of [7,4′-*OMe*-$^{13}C_2$]trimethoprim has been described (20), and [*m*-O$^{13}CH_3$]trimethoprim was synthesized essentially as described for the corresponding labeled brodimoprim (20). Samples contained ≈1 mM enzyme in 0.3 ml of buffer (500 mM KCl/50 mM potassium phosphate, pH 6.5), with 1 M equivalent of trimethoprim and, where required, NADPH.

NMR Spectroscopy. ¹H NMR spectra in 90% H_2O/10% ^2H_2O were obtained at 500 MHz (Bruker AM500 spectrometer) using the 1-1 pulse for water suppression (21, 22). ¹³C NMR spectra were obtained at 67.9 MHz (Bruker WH270 spectrometer); broad-band ¹H irradiation was applied by the composite pulse method (23).

Exchange Rate Measurements. Rates of exchange of N1-H with solvent H_2O were determined by analysis of the temperature dependence of the linewidth of the N1-H resonance of bound trimethoprim (24). The natural linewidth of this resonance in the ternary complex was assumed to be the same as that determined for the binary complex [linewidth ($W_{1/2}$) = 32 Hz at 298 K; activation energy (E_a) = 13 kJ/mol; ref. 24]. Data were obtained at five or six temperatures over the range 273–313 K (binary complex; ref. 24) or 293–323 K (ternary complex). Rates of exchange ("flipping") of the benzyl ring were estimated by comparing ¹³C spectra of [*m*-O$^{13}CH_3$]trimethoprim bound to the enzyme with line shapes simulated by using McConnell's (25) modification of the Bloch equations, assuming a linewidth in the absence of exchange of 7 Hz (as observed for the 4′-OCH_3 resonance of [7,4′-*OMe*-$^{13}C_2$]trimethoprim). Data were obtained at five or six temperatures over the range 273–293 K for the binary complex and 293–323 K for the ternary complex.

¹³C Relaxation Measurements. The spin-lattice relaxation rate (R_1) was measured by inversion recovery, the spin-spin relaxation rate (R_2) from the resonance linewidth, and the nuclear Overhauser effect (NOE) was measured by integration of the ¹³C resonance with and without ¹H irradiation. The ¹³C–¹H cross-relaxation rate (σ_{CH}) was estimated in two ways. First, it was calculated from R_1 and the NOE by using the relationship $\eta_{CH} = (\gamma_H/\gamma_C)(\sigma_{CH}/R_1)$. Second, independent values of R_1 and σ_{CH} were obtained from measurements of the build-up of the NOE as a function of the time of ¹H irradiation; these latter values are shown in parentheses in Table 2.

Analysis of Relaxation Measurements. The effects of molecular motion on the four measured relaxation parameters, R_1, R_2, σ_{CH}, and NOE are expressed in terms of the spectral densities, $\mathbf{J}(\omega)$, at the Larmor frequency for carbon or proton, or the sum or difference of these frequencies. For isotropic reorientation of the C–H vector, $\mathbf{J}(\omega)$ is given by

$$\mathbf{J}(\omega) = \frac{2}{5}\left(\frac{\tau_R}{1 + (\omega\tau_R)^2}\right), \quad [1]$$

where τ_R is the overall rotational correlation time. To incorporate the effects of internal motion, we use the approach of Lipari and Szabo (26, 27), with the spectral density function

$$\mathbf{J}(\omega) = \frac{2}{5}\left(\frac{\mathbf{S}^2\tau_R}{1 + (\omega\tau_R)^2} + (1 - \mathbf{S}^2)\tau_{int}\right). \quad [2]$$

The internal motion is characterized by two parameters: τ_{int}, an effective correlation time, and $\mathbf{S}^2$, the square of the order parameter for the motion. The order parameter is a measure of the amplitude of each motion, with $\mathbf{S}_i^2 = 1$ for complete immobilization and $\mathbf{S}_i^2 = 0$ for isotropic motion. [The derivation of Eq. 2 assumes that $\tau_{int} << \tau_R$ and $(\omega\tau_{int})^2 << 1$.]

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In considering motion of the 4′-OCH_3 relative to the 7-CH_2, we use the correlation time of the 7-CH_2 as τ_R and assume that the methyl group will be spinning freely about its symmetry axis, O-CH_3, which leads to $S^2_{obs} = 0.111$ (26, 27). If, in addition, there is rotation about either or both the C7-C1′ and C4′-O bonds (which are colinear), then $S^2_{obs} = S^2_{O\text{-}CH_3} \cdot S^2_{C4'\text{-}O} \cdot S^2_{C7\text{-}C1'}$; free rotation about these latter bonds gives $S^2_{obs} = 0.0105$. The expected limits to S^2_{obs} for the 4′-OCH_3 are thus $0.111 > S^2_{obs} > 0.0105$. If there is restricted diffusion about C7-C1′ and/or C4′-O, S^2_{obs} will have an intermediate value; the order parameter for motion of the symmetry axis of the methyl group will be given by (27):

$$S^2_{axis} = S^2_{C4'\text{-}O} \cdot S^2_{C7\text{-}C1'} = \tfrac{1}{4}(3\cos^2\beta - 1)^2 + \frac{3\sin^2\beta\sin^2\gamma_o}{\gamma_o^2}(\cos^2\beta + \tfrac{1}{4}\sin^2\beta\cos^2\gamma_o), \quad [3]$$

where β is the angle between O-CH_3 and C4′-O, and the motion is restricted to an amplitude of $\pm\gamma_o$.

Relaxation Mechanism. The analysis described above depends on the assumption that relaxation is exclusively dipolar. Assuming isotropic motion and dipolar relaxation, and using $r_{CH} = 1.1$ Å, the measured values of R_1 and R_2 for C7 give $\tau_R \geq 15.3$ ns and $\tau_R \leq 15.5$ ns, respectively, where the equalities apply if relaxation is 100% dipolar. The close similarity of these values shows that relaxation of C7 is >98% dipolar. (The alternative $\tau_R = 124$ ps, which is also consistent with the R_1 value, is not consistent with the measured NOE.) For the 4′-OCH_3, τ_{int} is calculated from σ_{CH} and R_1 is used, together with τ_R and τ_{int}, to calculate $S_i^2 \leq 0.079$ (again the equality applies for wholly dipolar relaxation). The values of τ_R, τ_{int}, and S_i^2 can then be used to calculate values for R_1 at 50.3 MHz. The experimental value ($1.16 \pm 0.15\ s^{-1}$; Bruker WM200) is not significantly different from that calculated assuming 100% dipolar relaxation ($1.25\ s^{-1}$); a lower limit of 85% dipolar relaxation at 67.9 MHz is estimated.

RESULTS AND DISCUSSION

Dynamics of the Diaminopyrimidine Ring. In the complex, trimethoprim is protonated on N1 of the diaminopyrimidine ring (24, 28, 29) and forms a hydrogen-bonding/charge–charge interaction with a carboxylate group of the protein (refs. 30 and 31; Asp-26 in the *L. casei* enzyme; see Fig. 1). The rate of exchange with solvent of the N1 proton involved in this hydrogen bond can be measured by analysis of the linewidth of its resonance, which has been identified in the ^1H spectrum of the *L. casei* enzyme (24). The measured rates of exchange in the binary and ternary complexes, together with their activation energies, are given in Table 1.

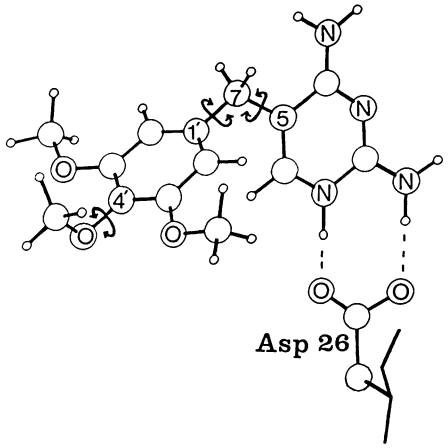

FIG. 1. The conformation of trimethoprim in its complex with *L. casei* dihydrofolate reductase (31, 33), together with its relationship to the carboxylate group of Asp-26. The atom numbering for the trimethoprim molecule is also indicated.

Hydrogen exchange in proteins can be considered to proceed by a two-step mechanism (8): an "opening" of the structure to allow access to a solvent molecule, followed by the chemical exchange process. Addition of imidazole, an effective catalyst of N-H exchange, to the enzyme–trimethoprim sample in concentrations as high as 0.2 M had no effect on the linewidth of the N1-H resonance, demonstrating that it is the opening rate—i.e., the rate of structural fluctuation—that determines the rate of exchange. The rate of the fluctuation is therefore 34 s^{-1} at 298 K, increasing to 250 s^{-1} at 318 K, and this must involve, as a minimum, breakage of the N1-H . . . Asp-26 hydrogen bond. The interaction between the pyrimidine ring and Asp-26 is thus breaking and re-forming at a rate more than an order of magnitude faster than that at which the whole drug molecule dissociates from the protein (32, 33). This must in turn involve movements of the pyrimidine ring and/or Asp-26, and the relatively high activation energy measured for the exchange does suggest that more than just a single hydrogen bond needs to be broken for exchange to occur.

Dynamics of the Trimethoxybenzyl Ring. Subnanosecond motion of the benzyl ring has been explored by ^{13}C relaxation measurements on [7,4′-*OMe*-$^{13}C_2$]trimethoprim in its complex with the enzyme. The measured relaxation parameters for the two enriched carbons are given in Table 2. Assuming the motion of the 7-CH_2 to be isotropic (Eq. 1), an effective rotational correlation time for this group can be calculated from its relaxation rates R_1 and R_2. Excellent agreement between the two estimates is obtained (see *Materials and Methods*), giving a value of 15.4 ± 1.5 ns at 295 K. This is very close to the value expected for the overall tumbling of a protein the size of dihydrofolate reductase (10).

By comparing the relaxation parameters of the 4′-OCH_3 to those of the 7-CH_2, we can obtain information on any rapid motions about the three intervening bonds: C7-C1′, C4′-O, and O-CH_3 (see Fig. 1). As described above, we have analyzed the data by the procedures of Lipari and Szabo (26, 27), in terms of (*i*) an effective correlation time for the internal motions affecting the 4′-OCH_3 and (*ii*) S_i^2, the square of the order parameters for motion about each of the three bonds linking it to C7.

The effective correlation time for internal motion, τ_{int}, can be calculated from σ_{CH}. For $0.0105 < S^2_{obs} < 0.111$ (see *Materials and Methods*), σ_{CH} is only weakly dependent on S^2, and we obtain $\tau_{int} = 4.3\ (\pm 0.8)$ ps. Having estimated the two correlation times τ_R and τ_{int} in Eq. 2, we can use the measured R_1, 0.81 s^{-1}, to calculate $S^2_{obs} = 0.079\ (\pm 0.012)$. This is significantly less than the value of 0.111 calculated by assuming that the only motion is the spinning of the methyl group about its axis, demonstrating that there is motion on the subnanosecond time scale about at least one of the other two bonds. With the present data, we cannot distinguish between motion about C7-C1′ and about C4′-O. However, we can set limits to the amplitudes of these motions, since

Table 1. Rates of N1-H exchange in trimethoprim bound to *L. casei* dihydrofolate reductase

	Complex	
	Enzyme–trimethoprim	Enzyme–trimethoprim–NADPH
k, s^{-1} (298 K)	34 (±3)	9 (±1)
k, s^{-1} (318 K)	250 (±20)	144 (±10)
E_a, kJ/mol	75 (±9)	113 (±13)

Data for the binary complex are from ref. 24.

Table 2. ^{13}C relaxation parameters of [7,4′-*OMe*-$^{13}C_2$]-trimethoprim bound to *L. casei* dihydrofolate reductase

	Carbon	
	7-CH_2	4′-OCH_3
R_1, s^{-1}	4.81	0.81 (0.86)
R_2, s^{-1}	120	22
^{13}C {^1H} NOE	1.19	1.78
σ_{CH}, s^{-1}	0.23	0.16 (0.14)

Values of R_1 and σ_{CH} in parentheses were obtained from measurements of the build-up of the NOE as a function of the time of ^1H irradiation. Experimental precision is estimated (from replicate determinations) as ±10% for the relaxation rates and ±17% for the NOE measurements.

$S^2_{C7\text{-}C1'}S^2_{C4'\text{-}O} = S^2_{obs}/S^2_{O\text{-}CH3} = 0.712\,(\pm 0.1)$. Thus, from Eq. 3, if there is motion about only one of the two bonds, its amplitude will be ±36°, while if there is motion of equal amplitude about both bonds, $S^2_{C7\text{-}C1'} = S^2_{C4'\text{-}O} = 0.843$, and the amplitude will be ±25°. These estimated amplitudes are toward the lower end of the range of amplitudes calculated from relaxation data for bonds in amino acid side chains of proteins (27, 34). Since the latter include surface residues, it is likely that the subnanosecond fluctuations in trimethoprim conformation are similar in magnitude to those of internal amino acid side chains.

Millisecond motion of the benzyl ring of trimethoprim has been studied by line-shape analysis of the ^{13}C resonances of [*m*-$O^{13}CH_3$]trimethoprim. When the molecule is bound to the enzyme, the environments of the two sides of the ring will be different, giving rise to two distinct *m*-methoxy resonances. As shown in Fig. 2, at 273 K two separate ^{13}C resonances are indeed observed for [*m*-$O^{13}CH_3$]trimethoprim bound to dihydrofolate reductase. However, as the temperature is increased, the two signals broaden and coalesce, until at 298 K a single sharp signal is observed, with a chemical shift almost exactly midway between those of the two signals seen at low temperature. This behavior is characteristic of an exchange process in which the benzyl ring of trimethoprim is able to rotate—or, more probably (11), "flip" by 180°—about its symmetry axis, thus interchanging the positions of the two *m*-methoxy groups and averaging their resonances. The values for the rate and activation energy of the flipping of the ring in the binary and ternary complexes, obtained by analysis of the temperature dependence of the line shape, are given in Table 3. Comparison with the measured dissociation rate constants (32, 33) shows that the rate of flipping is >100 times faster, so that this motion of the trimethoxybenzyl ring must take place in the bound state.

A simple 180° rotation of the benzyl ring of bound trimethoprim about this bond would not in fact lead to an identical structure, since the *p*-methoxy substituent is out of the plane of the ring (30, 31). Either the two states of the ring differ in the orientation of this *p*-methoxy group ("up" or "down"), in which case the equal amplitudes of the two ^{13}C signals observed at low temperature indicate that these two orientations are energetically equivalent, or there is a concerted rotation about both the C7-C1′ and the C4′-O bonds. We have recently (20) used line-shape analysis to determine the rate of flipping of the benzyl ring of brodimoprim, a trimethoprim analogue in which the *p*-methoxy group is replaced by a bromine atom and in which the complication of an out-of-plane substituent is therefore absent. The rate is somewhat slower (at 280 K, $k = 30\ s^{-1}$ vs. $80\ s^{-1}$ for trimethoprim) and the activation energy is somewhat greater (59 ± 9 kJ/mol; this is given incorrectly in ref. 20) for brodimoprim than for trimethoprim, so that the *p*-methoxy group clearly does not introduce any appreciable additional contribution to the rotational barrier.

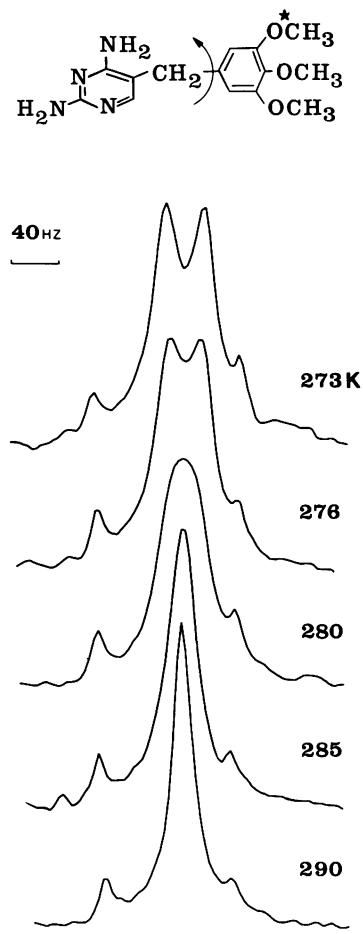

FIG. 2. The 67.9-MHz ^{13}C NMR spectra of [*m*-$O^{13}CH_3$]trimethoprim bound to *L. casei* dihydrofolate reductase as a function of temperature. The major resonances are those of the bound inhibitor; minor signals arise from a small amount of free trimethoprim and from natural abundance background resonances from the protein.

Similar flipping of a symmetrical aromatic ring has been observed for tyrosine and phenylalanine residues in proteins (11, 35, 36) and for other ligands bound to dihydrofolate reductase (15, 16). In each case, the observed barrier is made up of the intrinsic barrier to rotation about the relevant bond, here C7-C1′, and the barrier arising from steric interactions with the surrounding atoms in the complex as the ring flips. In the conformation that trimethoprim adopts when bound to the enzyme (Fig. 1), a major contribution to the barrier to rotation about C7-C1′ must arise from steric barriers within the trimethoprim molecule itself, due to unfavorably close contacts between C2′ or C6′ (and their attached hydrogen atoms) on the benzyl ring and C6 on the pyrimidine ring. Simple molecular mechanics calculations, considering only steric interactions within the trimethoprim molecule itself, give a calculated barrier to rotation about C7-C1′ of >2000 kJ/mol. This is clearly incompatible with the measured values of 40–70 kJ/mol, particularly since the latter include

Table 3. Rates of flipping of the trimethoxybenzyl ring about C7-C1′ in trimethoprim bound to *L. casei* dihydrofolate reductase

	Complex	
	Enzyme–trimethoprim	Enzyme–trimethoprim–NADPH
k, s^{-1} (298 K)	250 (±27)	15 (±2)
k, s^{-1} (318 K)	720 (±70)	80 (±9)
E_a, kJ/mol	42 (±4)	67 (±7)

contributions from interactions with atoms of the protein as well as those within trimethoprim. It follows that the flipping of the benzyl ring cannot take place at the observed rates if the trimethoprim is fixed in the conformation shown in Fig. 1. To avoid the steric clashes between C2′,6′ and C6, it is necessary to introduce a rotation about C5-C7 so as to change the mutual orientation of the two rings. The molecular mechanics calculations indicate that a rotation of at least ±60° about C5-C7 is required to bring the calculated barrier to rotation about C7-C1′ arising from intramolecular interactions down to the right order of magnitude. This rotation about C5-C7 must lead to a substantial movement of either the benzyl or the diaminopyrimidine ring. Examination of a model of the enzyme–trimethoprim complex (Fig. 3) shows that the diaminopyrimidine ring is bound in a relatively narrow slot, closely packed round with protein atoms, and that motion of the benzyl ring is much the more likely alternative. This clearly produces a major change in the overall shape of the trimethoprim molecule (Fig. 3), which would result in the favorable interactions of the trimethoxybenzyl ring with the protein being broken. In addition, in the "activated state" (Fig. 3 *Lower*) there are now some very unfavorable contacts with atoms of the protein that must be relieved by simultaneous fluctuations in the protein structure. The measured rates of ring flipping indicate that these changes in conformation occur with a frequency of 250 s^{-1} at 298 K.

Effects of Coenzyme Binding. In the presence of NADPH, trimethoprim binds 135 times more tightly to dihydrofolate reductase; this increased binding energy comes from changes in the interactions of both the diaminopyrimidine and the benzyl rings with the protein (39, 40). It is accompanied by a decrease by a factor of ≈2 in the rate of exchange of the N1-H, a decrease by a factor of 10 in the rate of flipping of the benzyl ring, and a substantial increase in the activation energies of these two processes (Tables 1 and 3).

Model building on the basis of the available crystal structures (30, 31, 37) indicates that the nicotinamide and ribose rings of the bound coenzyme are close to the benzyl ring of trimethoprim (20). The consequent changes in the environment of the benzyl ring are reflected in the substantial changes in ^{13}C chemical shift of the *m*-OCH_3 resonances on NADPH binding (from 0.44 and 0.96 ppm, relative to free trimethoprim, in the binary complex to 0.55 and 1.34 ppm in the ternary complex). Resonances of several other carbons of trimethoprim are also affected by NADPH binding (20); in the case of C6, the change in shift may reflect a small change in the relative orientation of the benzyl and pyrimidine rings (20). These direct effects may contribute to the observed decrease in the rate of benzyl ring flipping.

By contrast, the model building indicates that the bound coenzyme does not directly hinder solvent access to the N1-H of the pyrimidine ring. The observed increase in the barrier to N1-H exchange must arise from an inhibition of the fluctuations needed to allow the N1-H . . . Asp-26 hydrogen bond to break, either by the direct coenzyme–trimethoprim contacts described above or by a coenzyme-induced conformational change. As in the case of the enzyme–methotrexate complex (41), there may be a movement of helix C (residues 42–49) produced by coenzyme binding (41, 42).

Conclusions. It is clear that fluctuations in the protein and ligand structure are important both in the kinetics of ligand binding (43, 44) and in the equilibrium binding energy (45). In the present work, we have shown that there are relatively large-scale fluctuations in the conformation of trimethoprim bound to dihydrofolate reductase. The N1-H exchange and the flipping of the benzyl ring occur at broadly similar rates in the millisecond range. However, comparison of the measured rates and activation energies shows that the fluctuations involved in the two processes are not identical, and this is confirmed by the different effects of the coenzyme on the two. The structural fluctuations that result in disrupting the interactions with the protein of, on the one hand, the benzyl ring and, on the other, the diaminopyrimidine ring may have elements in common but appear to be largely independent. Indeed, it seems likely that when these two kinds of fluctuation do happen to occur simultaneously the trimethoprim molecule will dissociate from the enzyme. The possible role

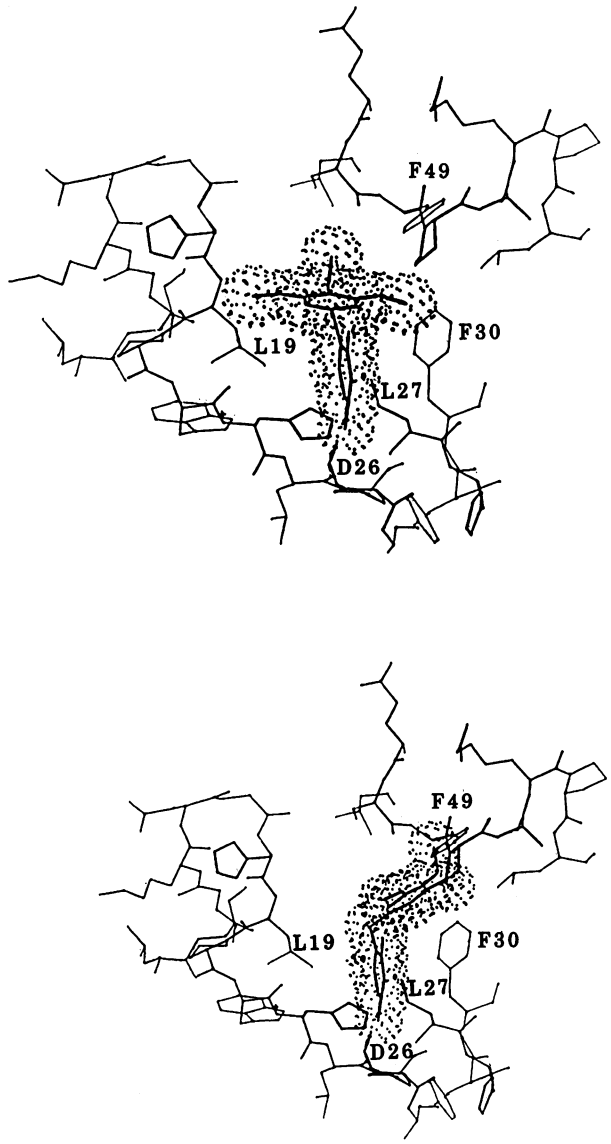

FIG. 3. The active site region of the *L. casei* dihydrofolate reductase–trimethoprim complex; the van der Waals surface of the trimethoprim molecule is indicated by dots. (*Upper*) The equilibrium conformation of the complex, derived by model building from the crystal structures of the methotrexate complex of the *L. casei* enzyme (37) and the trimethoprim complex of the *Escherichia coli* enzyme (30, 31). The conformation is defined by τ(C6-C5-C7-C1′) = 190°, τ(C5-C7-C1′-C2′) = 70°. (*Lower*) Derived from *Upper* by a rotation of 65° about the C5-C7 bond of trimethoprim to give τ(C6-C5-C7-C1′) = 255°. Molecular mechanics calculations (including only terms relating to van der Waals interactions between atoms within the trimethoprim molecule, and using a value of 117.9° for the bond angle C5-C7-C1′, as observed in crystals of a number of trimethoprim analogues; e.g., see ref. 38) indicated that this is the minimum rotation about C5-C7 required to bring the calculated barrier to rotation about C7-C1′ down to below 200 kJ/mol (in this conformation, the calculated barrier is 42 kJ/mol). Note that in this figure no adjustments have been made to the protein structure to relieve steric clashes with atoms of the trimethoprim molecule in its altered conformation.

of conformational rearrangements of the ligand in the kinetics of binding—the "zipper" mechanism—has been discussed earlier (44) and shown to be a means by which a high degree of conformational specificity can be reconciled with rapid association and dissociation. This mechanism implies that a degree of conformational flexibility of the drug–receptor complex may be functionally important.

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