Characterization of Rates of Ring-Flipping in Trimethoprim in Its Ternary Complexes with *Lactobacillus casei* Dihydrofolate Reductase and Coenzyme Analogues†

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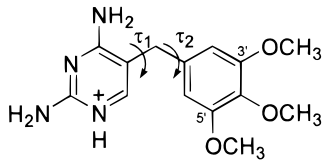
ABSTRACT: NMR measurements have been used to investigate rates of ring-flipping and the activation parameters for the trimethoxybenzyl ring of the antibacterial drug trimethoprim (TMP) bound to *Lactobacillus casei* dihydrofolate reductase (DHFR) for a series of ternary complexes formed with analogues of the coenzyme NADPH. Rates were obtained at several temperatures from line shape analyses (^{13}C-edited HSQC ^{1}H spectra) and transfer of magnetization measurements (zz-HSQC) on complexes containing 3′-*O*-[^{13}C]trimethoprim. Examination of the structures of the complexes indicates that ring-flipping can only be achieved following major conformational changes and transient fluctuations of the protein and coenzyme structure around the trimethoxybenzyl ring. There is no simple correlation between rates of ring-flipping and binding constants. The presence of the coenzyme nicotinamide ring (in either its reduced or its oxidized forms) in the binding site close to the trimethoxybenzyl ring moiety is the major factor reducing the ring-flipping on coenzyme binding. Thus, the ternary complex with NADPH shows the largest reduction in the rate of ring-flipping (11 ± 3 s^{-1} at 298 K) as compared with the binary complex (793 ± 80 s^{-1} at 298 K). Complexes with NADPH analogues that either have no nicotinamide ring or are known to have their nicotinamide rings removed from the binding site show the smallest reductions. For the DHFR·TMP·NADP$^+$ complex where there are two conformations present, very different rates of ring-flipping were observed for the two forms. The activation parameters ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) for the ring-flipping in all the complexes are discussed in terms of the protein−ligand interactions and the possible constraints on the pathway through the transition state.

NMR[1] has proved to be an excellent technique for measuring dynamic processes in proteins. Most of these studies have concentrated on characterizing the very rapid motions (10^8−10^{12} s^{-1}) that are accessible via relaxation time measurements (*1*). However, there is an increasing interest in determining the rates of much slower intramolecular conformational interconversions (10^0−10^4 s^{-1}) where information about the dynamic processes could make important contributions to understanding the function of macromolecular systems. In protein−ligand complexes, these slow dynamic processes can be either interconversions between different conformational states or interconversions between two identical conformational states involving hindered rotations about single bonds. We have encountered several examples of both types of such behavior in our studies of antifolate drugs bound to *Lactobacillus casei* dihydrofolate reductase (*2*−*9*). In some complexes, correlated rotations about two single bonds have been detected (*3*, *10*−*12*). If the rates of rotation about two collinear single bonds are the same, this provides a mechanism for allowing a part of the protein or ligand to rotate independently of the remainder of the complex while maintaining many of the protein−ligand interactions. However, rotation about one single bond is often sufficient to allow a fragment of the complex to rotate (*7*, *8*), and this is the case for the aromatic ring-flipping in complexes with trimethoprim (Scheme 1) (*5*, *6*). In some cases, the possible dynamic pathways for the ring-flipping have been calculated (*13*).

Scheme 1: Trimethoprim



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The availability of isotopically labeled (^{13}C, ^{15}N, and ^{19}F) ligands and proteins has facilitated the measurements of these dynamic processes. The early measurements were made by

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[1] Abbreviations: DHFR, dihydrofolate reductase; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; HSQC, heteronuclear single quantum coherence spectroscopy; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PADPR-OCH$_3$, methyl β-riboside of 2′-phosphoadenosine 5′-(diphosphoribose); TMP, trimethoprim; 3D, three-dimensional; 2D, two-dimensional; zz-HSQC, zz-magnetization exchange HSQC spectroscopy.

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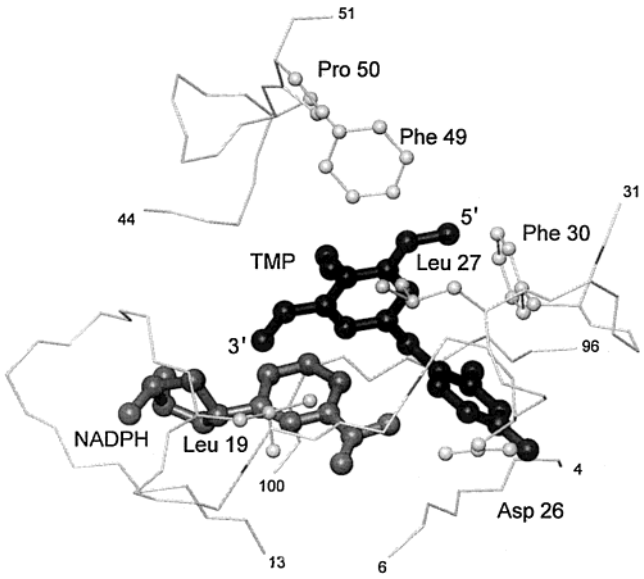

FIGURE 1: Part of the solution structure of the ternary complex DHFR·TMP·NADPH showing the locations of the drug and the coenzyme in their binding sites.

directly detecting the heteronuclei and measuring either their line shapes or magnetization transfer behavior (1D and 2D exchange methods) as a function of temperature. In the present study, we have used editing methods of detection to examine ^{1}H nuclei attached to ^{13}C to provide improved sensitivity. For example, we have used HSQC-based experiments to determine the rates of ring-flipping of the trimethoxybenzyl ring of 3′-O-[^{13}C]trimethoprim in a series of trimethoprim-containing ternary complexes formed with *L. casei* dihydrofolate reductase and various coenzyme analogues. Structural information is available for several of these complexes. The detailed structures of the binary DHFR·TMP and ternary DHFR·TMP·NADPH in solution have been determined previously (*14*; Polshakov, Birdsall, and Feeney, unpublished results). The relative positions of trimethoprim and NADPH and some of their binding site residues in the DHFR·TMP·NADPH complex are shown in Figure 1. The trimethoprim trimethoxybenzyl moiety is seen to be near to the reduced nicotinamide ring of NADPH. The groups in closest proximity are the C7 methylene group of TMP and the C4 position of the NADPH nicotinamide ring.

The ternary complex DHFR·TMP·$NADP^+$ has also been extensively studied by NMR and found to exist in two different conformational states that interconvert at a rate of 6 s^{-1} at 304 K (*4*, *6*, *15*). The two states were found to differ mainly in the orientations of the nicotinamide ring of bound $NADP^+$, which in one form (form I) is buried *in* the protein and occupies essentially the same site as the reduced nicotinamide ring in the complexes with NADPH whereas in the other form (form II) the nicotinamide ring extends *out* of the protein complex into free solution. There are also differences in the nicotinamide ribose and the pyrophosphate backbone conformations in the two forms.

The binding constants and cooperativity factors for the complexes examined in this study have already been determined (see Table 1), and these provide useful background information for a systematic study of ring-flipping in bound trimethoprim. A complete understanding of the ligand binding will obviously include not only information about the factors causing the specific interactions but also some information about any dynamic processes in the complex. While these latter processes will clearly be implicated in the association and dissociation rates, their influences on the binding specificity and cooperativity of binding will be more difficult to assess.

MATERIALS AND METHODS

Materials. L. casei DHFR was expressed in *Escherichia coli* containing the *L. casei* DHFR gene, and the protein was isolated and purified as described earlier (*18*, *19*). NADPH and $NADP^+$ were obtained from Sigma and used without further purification. PADPR-OCH_3 (a coenzyme analogue in which the nicotinamide ring is replaced stereospecifically by a methoxy group) was prepared by the calf spleen NAD^+ glycohydrolase-catalyzed methanolysis of $NADP^+$ (*20*) and purified on a column of diethylaminoethylcellulose (Whatman DE-52). [3′-^{13}C]Trimethoprim was supplied by Dr. Levshine (Center for Drug Chemistry, Moscow) and was synthesized by the well-established condensation reaction of propionitrile with [3-^{13}C]-3,4,5-trimethoxybenzaldehyde (*21*, *22*). The latter compound was obtained by alkylation of 3,4-dimethoxy-5-hydroxybenzaldehyde using ^{13}C-labeled methyl iodide. This results in only one of the *m*-methoxy group of the TMP being labeled with ^{13}C, and this corresponds to a 50% labeling in each of the 3′ and 5′ positions. The synthesized ^{13}C-labeled trimethoprim was characterized by mass spectrometry and NMR spectroscopy. After recrystallization from ethanol, the sample contained at least 98% of the principal product.

Samples of equimolar ligand−enzyme complexes (concentration, 2.5 mM) of ^{13}C-labeled TMP·DHFR, ^{13}C-labeled TMP·DHFR·NADPH, ^{13}C-labeled TMP·DHFR·$NADP^+$, and ^{13}C-labeled TMP·DHFR·PADPR-OCH_3 were prepared in D_2O and 50 mM potassium phosphate with 100 mM KCl, pH* = 6.5 (the pH* values being meter readings, unadjusted for deuterium isotope effects).

NMR Experiments. NMR experiments were performed over the temperature range of 278−313 K on a Varian UNITY Plus 500 MHz spectrometer. The temperature calibration was verified using standard samples of methanol and ethylene glycol. 1D $^{1}H-^{13}C$ HSQC experiments were used to detect resonances of protons directly attached to ^{13}C nuclei, and 2D zz-HSQC experiments were used to measure rates of some of the exchange processes. The latter experiments employed the pulse sequence of Yamazaki and co-workers (*23*) with minor modifications as described earlier (*11*). The experiments were carried out at several different temperatures with a series of different mixing times (2−64 ms) being used at each temperature and with acquisition times of 32 and 150 ms in the ^{13}C and ^{1}H dimensions, respectively. A 2D NOESY−HSQC spectrum was recorded at 298 K on the ^{13}C-labeled TMP·DHFR·NADPH sample using a modified version of the original sequence of Marion and co-workers (*24*) with a 50-ms mixing time and acquisition times of 28 and 150 ms in the ^{13}C and ^{1}H dimensions.

All the NMR experiments used the Watergate sequence for water suppression (*25*) and the GARP sequence (*26*) for ^{13}C and ^{15}N decoupling during the detection period. Phase-sensitive detection in the indirect dimensions was obtained using the method of States and co-workers (*27*). The spectra

Table 1: Rates and Thermodynamic Parameters for the Trimethoxybenzyl Ring-Flipping of Trimethoprim in Different Complexes with *L. casei* DHFR and the Relative Trimethoprim Association Binding Constants

complex	rate (s^{-1}) 278 K	rate (s^{-1}) 298 K	$\Delta H^{\ddagger}$ (kJ/mol)	$T\Delta S^{\ddagger}$ (kJ/mol)[a]	K[b]
DHFR·TMP[c]	119 ± 20	793 ± 80	63 ± 1	8 ± 1	1[d]
DHFR·TMP·PADPR-OCH_3[c]	68 ± 10	469 ± 80	64 ± 1	8 ± 1	4.7
DHFR·TMP·NADPH[e]	0.9 ± 0.5[f]	11 ± 3	81 ± 5	15 ± 2	135
DHFR·TMP·$NADP^+$ (out)[c]	23 ± 5	191 ± 30	71 ± 3	13 ± 2	5
DHFR·TMP·$NADP^+$ (in)[e]	1.9 ± 0.5	21 ± 5	109 ± 5	45 ± 3	0.8

[a] Measured at 298 K. [b] K is the ratio of the TMP association constants in the ternary complex as compared to the binary DHFR·TMP complex (*4, 16, 17*). [c] Measured by line shape analysis of the 1D ^{1}H HSQC spectra. [d] Association binding constant for trimethoprim binding to *L. casei* DHFR is 2.0×10^7 M^{-1} (*16, 17*). [e] Measured by 2D zz-HSQC exchange experiments. [f] This value was estimated from the corresponding values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

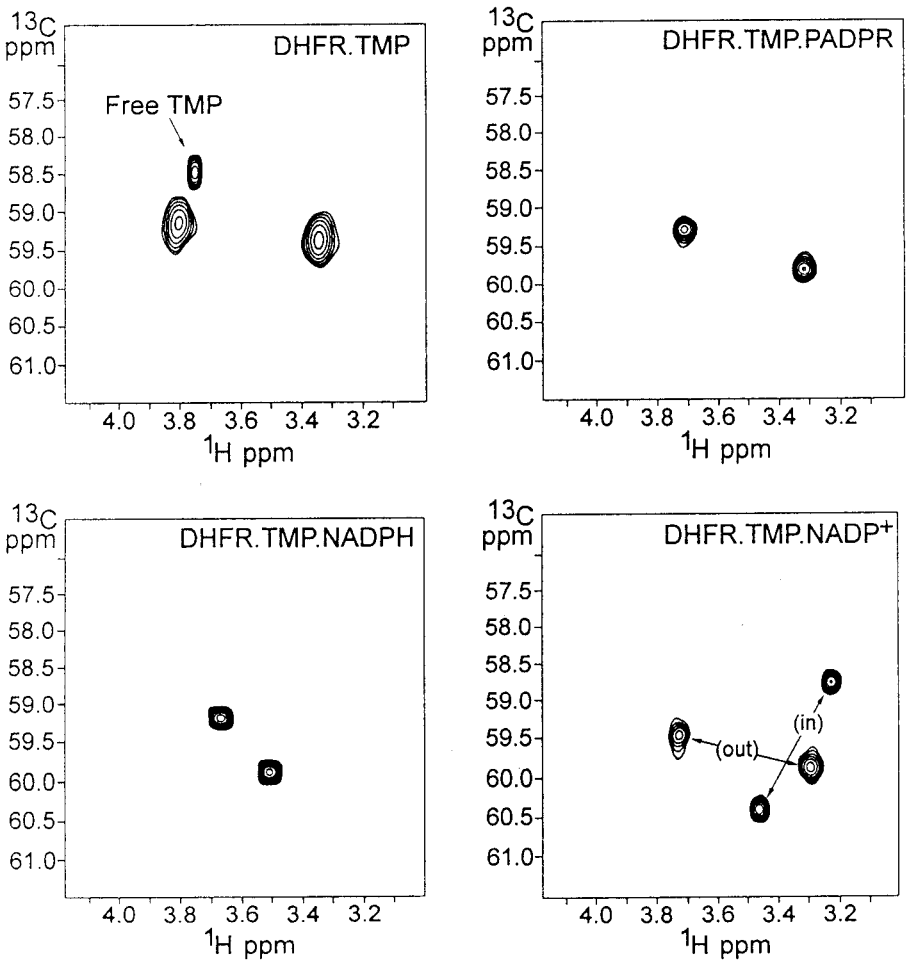

FIGURE 2: $^{13}C-^1H$ HSQC spectra for the complexes DHFR·TMP, DHFR·TMP·PADPR-OCH_3, DHFR·TMP·NADPH, and DHFR·TMP·$NADP^+$ formed with ^{13}C-labeled trimethoprim recorded at 278 K. The DHFR·TMP·$NADP^+$ complex exists in two forms; one has the nicotinamide ring of $NADP^+$ inside the binding site of the enzyme (in) and the other has this ring outside the protein (out).

were processed and displayed using VNMR (Varian) and FELIX (Molecular Simulations Inc.) The ^{1}H chemical shifts were measured from dioxane as an internal reference and then referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), where dioxane has a chemical shift of 3.75 ppm.

Characterization of Ring-Flipping. At low temperature (<283 K), the trimethoxybenzyl ring of bound trimethoprim is flipping about the C7−C1′ (τ_2 torsion angle) bond sufficiently slowly for two separate signals to be observed for the 3′- and 5′-OCH_3 groups. This is seen clearly in the $^1H-^{13}C$ HSQC spectrum of the DHFR·TMP complex shown in Figure 2. Because of the wide range of ring-flipping rates encountered in the various complexes over the temperature range studied, it was necessary to use a combination of line shape analysis and magnetization transfer zz-HSQC experiments to extract all the data. The rate constants derived from the zz-HSQC spectra were obtained by measuring the normalized intensities of cross-peaks I:

$$I = \sum(\text{cross-peak volume})/(\sum(\text{cross-peak volume}) + \sum(\text{auto-peak volume})) \quad (1)$$

As shown earlier (*28*) for two-site exchange, if the cross-relaxation during the mixing time can be neglected, the normalized cross-peak intensity is a function of the mixing

time τ_m and has the following form:

$$I(\tau_m) = 0.5[1 + \exp(-2k\tau_m)] \quad (2)$$

where k is the rate constant of the exchange process. Values of k with their standard deviations were calculated by nonlinear fitting of the normalized intensities measured at different mixing times. The enthalpies and entropies of activation were calculated by nonlinear fitting of values of k, obtained at different temperatures (from 283 to 313 K), using the Eyring equation (*29*) from the absolute rate theory:

$$k = Kk_BT/h \exp((\Delta H^{\ddagger} - T\Delta S^{\ddagger})/RT) \quad (3)$$

where k_B and R are the Boltzmann and gas constants, respectively; and K is the transmission coefficient, which was taken as 0.5 in all calculations. This is a good approximation for a ring-flipping process because the probabilities of molecules in the transition state reaching their final and initial states are the same (as these states are identical).

Values of the enthalpy and entropy of activation of ring-flipping together with the rate constants for ring-flipping were also determined from a full line shape analysis of a series of 1D HSQC spectra. Values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were obtained by Powell minimization of the penalty function χ^2 calculated for a series of spectra recorded at different temperatures:

$$\chi^2 = \sum_i \sum_j (A_i I(\omega_j + B_i)^{calc} - I(\omega_j)^{exp})^2 \quad (4)$$

In eq 4, $I(\omega_j)^{exp}$ is the experimentally measured intensity at a frequency point ω_j in the 1D HSQC spectrum recorded at the ith temperature, $I(\omega_j)^{calc}$ is the theoretical intensity for ω_j calculated using the standard equations for a two-site exchange process (*29*), A_i and B_i are parameters used to adjust the vertical and horizontal scales when comparing experimental and calculated spectra. Rate constants for the exchange process used in the $I(\omega_j)$ calculations were obtained using eq 3. Eight spectra were recorded for each complex at a series of temperatures between 278 and 313 K (with steps of 5 K), and normally 200−300 frequency points were used for fitting the principal part of each spectrum. The natural line widths of the exchanging signals at 298 K were also obtained by fitting χ^2. At temperature T, the natural line width (LW) was estimated using the following equation:

$$1/LW^T = 1/LW^{298} \exp[-E_v/R(1/T - 1/298)] \quad (5)$$

where E_v is the activation energy for viscosity, and this parameter was also obtained by Powell minimization of the χ^2 penalty function. The calculated activation energy for viscosity, E_v, for all the samples had a value of 22 ± 2 kJ/mol, which is in good agreement with typical values for protein solutions (*30*, *31*).

It should be noted that the rate of ring-flipping in the DHFR·TMP binary complex measured in this work at 298 K (∼790 s^{-1}) is about 3 times higher than that reported earlier (250 s^{-1} (*5*)). The errors in the line shape analysis of the earlier data arose because of the small difference in the ^{13}C chemical shifts between the individual signals (27 Hz at 67.9 MHz) and the low temperature of coalescence. In this work, we make use of the much larger chemical shift difference in the ^1H dimension (235 Hz at 500 MHz), and the exchange rates can thus be measured over a larger range of temperature than in the previous studies. In the earlier work, the coalescence of the 3′- and 5′-OCH_3 ^{13}C signals of TMP in the binary complex DHFR·TMP occurred at ∼280 K, whereas in the present work, a temperature of 302 ± 1 K was needed to achieve coalescence of their ^1H signals. The change in line broadening due to the change of viscosity of the solution becomes substantial at lower temperatures, and this was not taken into account in the earlier work.

The sample ^{13}C-labeled TMP·DHFR·$NADP^+$, which has two slowly interconverting conformers (forms I and II), required a combination of line shape analysis and zz-HSQC experiments for extracting the dynamic parameters. For form I, it was necessary to use zz-HSQC experiments to obtain the rates of ring-flipping at temperatures below 308 K. These rates were then used to provide starting values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for form I for its line shape analysis. Form II could be examined by line shape analysis at all temperatures used. The complete spectrum (Figure 3), containing signals from both form I and form II, was fitted by summing the contributions from each exchanging pair to obtain the total signal intensity, neglecting at this stage any exchange between the two conformers that is known to be slow (6 s^{-1} at 304 K (*2*)). Finally, the results obtained were verified by calculating the NMR line shape for a four-site exchange process, including the rates of interconversion between form I and form II using the general multiple site exchange matrix algorithm (*29*). All the calculations and graphical representations of the results were obtained using the program Muses (*mu*ltiple *s*ite *e*xchange *s*imulations), written in-house.

RESULTS

Rates of Ring-Flipping and Activation Parameters. The ring-flipping dynamics of the trimethoxybenzyl ring of trimethoprim bound to *L. casei* dihydrofolate reductase have been characterized in several binary and ternary complexes: DHFR·TMP, DHFR·TMP·PADPR-OCH_3, DHFR·TMP·NADPH, and DHFR·TMP·$NADP^+$. At 278 K, the ^{13}C NMR spectra of all these complexes show separate signals for 3′- and 5′-OCH_3 groups, indicating that the ring-flipping is slow on the NMR time scale (Figure 2). The signals are rather sharp at this temperature for all the complexes except the binary complex, DHFR·TMP, where the signals from bound trimethoprim show substantial line broadening due to exchange. As the temperature is raised (spectra not shown), the signals for the binary complex broaden further until they coalesce in the ^1H dimension at 302 ± 1 K. The coalescence temperature in the ^{13}C dimension is lower (∼280 K) (*6*) because the frequency separation of the ^{13}C signals for this complex is approximately 6 times smaller than in the ^1H dimension. We therefore used ^1H 1D HSQC spectra recorded over the range 278−318 K to monitor the exchange processes. Subsequent full line shape analysis of the spectra allowed us to determine the thermodynamic parameters of ring-flipping (see Materials and Methods and Table 1). The spectra of the ternary complex DHFR·TMP·PADPR-OCH_3 show similar behavior for the 3′- and 5′-OCH_3 ^1H signals and coalesce at approximately the same temperature (299 K) as the binary complex. In contrast, the ^1H signals in the spectra of the ternary DHFR·TMP·NADPH are not coalesced

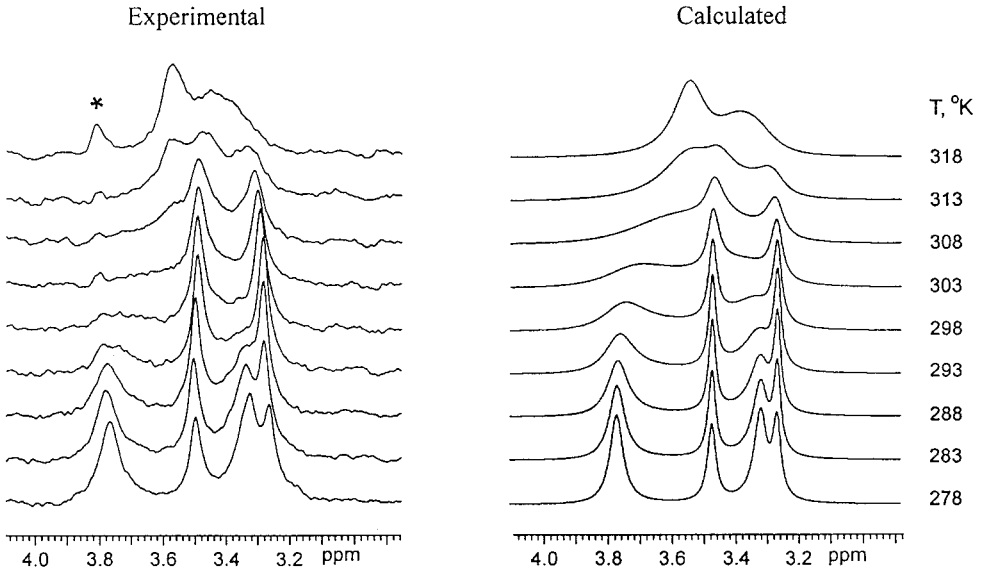

FIGURE 3: Experimental and calculated ^{1}H spectra of the ^{13}C-labeled TMP bound to *Lactobacillus casei* dihydrofolate reductase in its complex with NADP$^+$. The calculated spectra were derived from the best-fit parameters ($\Delta H^‡$and $\Delta S^‡$) shown in Table 1. For each pair of experimental and calculated spectra, the experimental temperature is indicated. At 318 K, a signal from some free ^{13}C-labeled TMP is observed in the spectrum (marked with an asterisk) due to partial thermal degradation of the enzyme and the subsequent release of ligand. The resonances at 3.25 and 3.49 ppm correspond to form I (in) of the ternary complex (in which the nicotinamide ring of NADP$^+$ is inside the binding site); the resonances at 3.75 and 3.32 ppm correspond to form II (out) (in which the nicotinamide ring of NADP$^+$ is on the surface of the enzyme).

Table 2: ^{1}H and ^{13}C Chemical Shifts and $^1J(^{13}C,^1H)$ Coupling Constants Measured for Methoxy Groups of Trimethoprim in Complexes with *L. casei* DHFR

	3′-OCH$_3$			5′-OCH$_3$			4′-OCH$_3$[a]
	δ (ppm)			δ (ppm)			δ (ppm)
complex	^{13}C	^{1}H	^1J (Hz)	^{13}C	^{1}H	^1J (Hz)	^{13}C
DHFR·TMP	59.1	3.82	146 ± 1	59.4	3.35	146 ± 1	61.6
DHFR·TMP. PADPR-OCH$_3$	59.2	3.74	147 ± 1	59.8	3.34	145 ± 1	61.8
DHFR·TMP·NADPH	59.2	3.69	146 ± 1	59.9	3.53	144 ± 1	61.8
DHFR·TMP·NADP$^+$ (out)	59.4	3.75	145 ± 2	59.9	3.32	145 ± 2	61.6
DHFR·TMP·NADP$^+$ (in)	58.7	3.25	146 ± 1	60.4	3.49	145 ± 2	61.9

[a] Chemical shifts for 4′-OCH$_3$ are taken from ref *6*.

at 318 K, the highest temperature studied. In this case, we had to use the 2D zz-HSQC experiment to measure the rates of ring-flipping (see Materials and Methods). For the DHFR·TMP·NADP$^+$ complex where there are two coexisting conformational forms, the coalescence temperature for form II (out) was 308 K and the ring-flipping rates could be obtained from line shape analysis at all temperatures studied, whereas for form I (in) the coalescence temperature is higher and a combination of zz-HSQC and line shape analysis was used to obtain the rates and activation parameters (see Materials and Methods).

The fastest rate of ring-flipping of the trimethoxybenzyl ring of TMP is found for the binary complex DHFR·TMP, and the slowest rate is found for the ternary complex DHFR·TMP·NADPH. The binary complex has the smallest TMP binding constant, and the ternary complex has the largest in the series of complexes examined here (*16*, *17*). However, a more general comparison of the ring-flipping rates with the trimethoprim binding constants (see Table 1) reveals that there is no simple correlation of the rates of ring-flipping and the binding constants (see Discussion). The two ternary complexes with the slowest rates have a nicotinamide ring close to the trimethoxybenzyl moiety (DHFR·TMP·NADPH and form I of DHFR·TMP·NADP$^+$ (in)) although they have very different trimethoprim binding constants. These complexes also show distinctive differences in their $\Delta H^‡$ and $\Delta S^‡$ activation parameters as compared to the binary DHFR·TMP complex.

Chemical Shifts and Coupling Constants in the Bound Trimethoprim. Table 2 contains the values of the ^{1}H and ^{13}C chemical shifts and the $^1J(^{13}C,^1H)$ coupling constants measured for the methoxy groups of trimethoprim in the complexes studied.

The locations of the 3′ and 5′ methoxy groups of the bound TMP within the binding site were determined from analysis of the 2D HSQC−NOESY data. Figure 4 shows part of the ^{1}H−^{13}C NOESY−HSQC spectrum for the DHFR·TMP·NADPH complex recorded at 298 K. The 3′-OCH$_3$ group was defined as the one closer to the loop comprising residues 9−23 (with NOEs to His18 Hα and Leul9 Hγ and Hδ1), and the 5′-OCH$_3$ group was defined as the one closer to helices B and C (with NOEs to Phe30 Hϵ and Hζ and Phe49 Hϵ).

The largest difference in the ^{1}H chemical shift between the binary TMP complex and the ternary complexes is observed for the 3′-OCH$_3$ in form I of the DHFR·TMP·

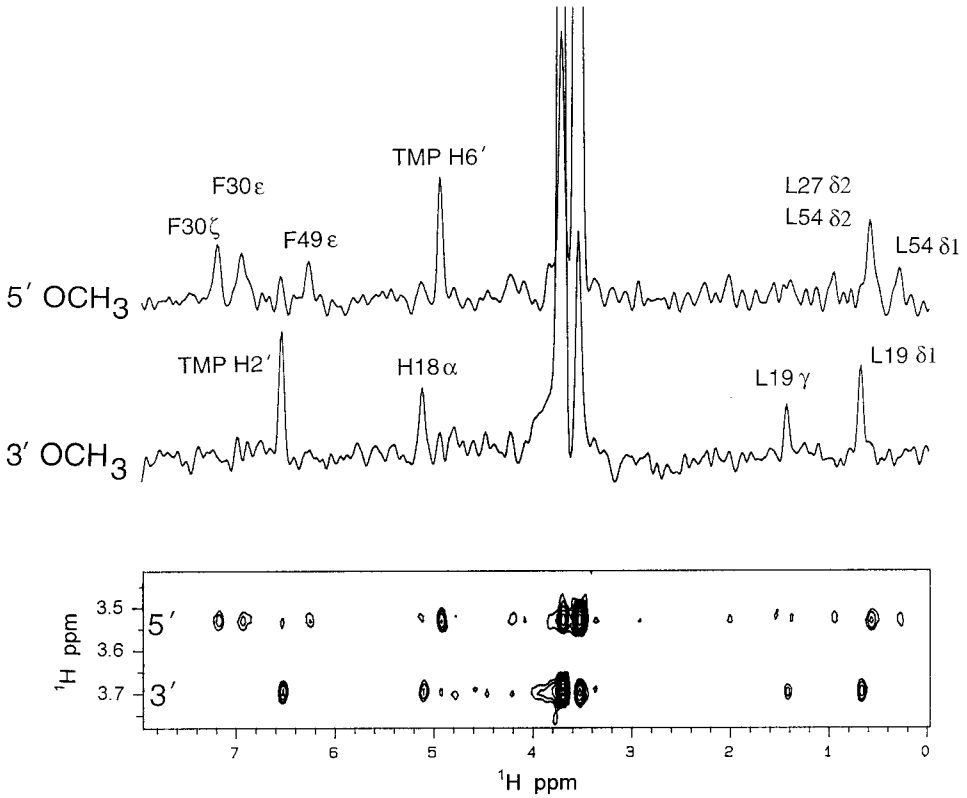

FIGURE 4: Part of the $^{13}C-^{1}H$ NOESY−HSQC spectrum of the ^{13}C-labeled TMP·DHFR·NADPH complex recorded at 298 K and used for the assignment of the 3′-OCH_3 and 5′-OCH_3 signals of TMP. By definition, the 3′-OCH_3 group is the one that is closer to His18, and the 5′-OCH_3 group is closer to Phe49 and Phe30.

$NADP^+$ complex (0.57 ppm). An effect of similar size is observed also for the ^{13}C signals of 3′-OCH_3 (0.4 ppm). The increased shieldings of ^1H and ^{13}C for the 3′-OCH_3 in form I of the DHFR·TMP·$NADP^+$ complex are due to the ring current shielding effects arising from the aromatic nicotinamide ring of the oxidized coenzyme that is situated with its plane over the 3′-OCH_3 group. This conclusion is supported by the fact that such an effect is not seen for the other ternary complexes: in the complex with PADPR-OCH_3 there is no nicotinamide ring; in the complex with NADPH, the nicotinamide ring is not aromatic; and in form II of the complex with $NADP^+$, the nicotinamide ring is oriented away from TMP binding site. The solution structure of DHFR·TMP·NADPH reveals the position of the nicotinamide ring with respect to the trimethoprim (Figure 1). If we assume that the reduced and oxidized nicotinamide rings in complexes with NADPH and $NADP^+$ occupy the same space in the binding site, then the nicotinamide ring is correctly positioned to induce the observed ring-current effect on 3′-OCH_3. X-ray results on other DHFR complexes containing NADPH and $NADP^+$ have shown that the reduced and oxidized nicotinamide rings occupy the same binding site (*32*).

For the 5′-OCH_3 group, the most significant differences in chemical shifts between the binary DHFR·TMP complex and the ternary complexes are observed for complexes formed with NADPH and $NADP^+$ (form I). These shielding differences could reflect slightly different relative dispositions of the trimethoxybenzyl ring of TMP in its binary and ternary complexes. Even small displacements of the trimethoxybenzyl ring away from the nicotinamide ring could result in changes in shielding caused by the surrounding residues such as Phe30, Phe49, and Pro50. The decrease in ^{13}C shielding for the 5′-OCH_3 groups in all ternary complexes as compared with the binary possibly reflects steric effects.

DISCUSSION

The eventual understanding of the kinetic and thermodynamic aspects of protein−ligand interactions will require detailed analyses of the rates and activation parameters for relevant dynamic processes in the bound ligand. Relatively slow conformational interconversions, operating over time scales of 10^0-10^4 s^{-1}, are likely to be important in these interactions, and some of these dynamic processes can be investigated rather easily using NMR techniques.

A typical example of such a process is provided by the ring-flipping of the trimethoxybenzyl ring of trimethoprim bound to DHFR. It is already known that the rate of the ring-flipping is not related in a simple way to the structural environment around the aromatic ring (*5*). Examination of the binding site in the DHFR·TMP complex reveals that there are several bulky groups in close proximity to the trimethoxybenzyl ring that would make it impossible for ring-flipping to take place without some substantial changes in the protein structure. Several theoretical studies (*33*−*36*) of closely related ring-flipping behavior for the aromatic rings of phenylalanine and tyrosine residues in proteins have shown that the low observed barriers to ring-flipping require that the protein structure around the aromatic ring must undergo rapid transient displacements that 'relax' the structure, thus relieving the unfavorable steric interactions hindering the ring-flipping. Such transient fluctuations in the protein structure around the trimethoxybenzyl ring of bound trimethoprim would similarly need to be present in order to allow ring-flipping in the DHFR·TMP complex. The observed

barriers to rotation will have contributions from the intrinsic rotation barrier about the C7−C1′ bond (τ_2 torsion angle) from protein−ligand steric hindrances and from protein−ligand attractive interactions that need to be broken in order for ring-flipping to occur. Although the intrinsic barrier to rotation is fairly low in free trimethoprim, it is very high in bound trimethoprim (*5*). In the conformation adopted by the bound trimethoprim there are major contributions to the barrier from steric interactions within the trimethoprim molecule itself (close steric clashes between the pyrimidine ring C6 and the benzyl ring C2′ and C6′ and their attached protons). Earlier molecular mechanics calculations (*5*) indicated, that in order to avoid these steric clashes, it is necessary for the τ_1 torsion angle about C5−C7 to change by at least ±60° in order to reduce the barrier to a sufficiently low value to allow ring-flipping to occur about the C7−C1′ bond (τ_2 torsion angle) at 298 K. This required rotation about the C5−C7 bond (τ_1 torsion angle) involves a substantial reorganization of the parts of the protein structure in contact with the benzyl and/or pyrimidine rings. Thus, the rate of ring-flipping is in fact reporting on the overall rate of a complicated series of conformational rearrangements rather than on a simple ring-flipping process. These major changes in the overall shape of the trimethoprim and protein that result in the breaking of favorable interactions between the ligand trimethoxylbenzyl ring and the protein have been discussed by Searle and co-workers (*5*).

As one can see from Table 1, complexes examined in this study can be divided into two groups according to the rate of ring-flipping. The first group contains the ternary complexes DHFR·TMP·NADPH and form I (in) of DHFR·TMP·$NADP^+$, where the trimethoxybenzyl ring of TMP undergoes slow ring-flipping. The remaining three TMP-containing complexes are characterized by much faster ring-flipping. There is no simple correlation between the rates of ring-flipping and the trimethoprim binding constants in the different complexes (see Table 1). For example, in the TMP·NADPH complex, where the binding constant for TMP is increased more than one 100-fold as compared to the binary complex, the rate of ring-flipping is much reduced, while in the TMP·$NADP^+$ (in) complex, where the TMP binding is not increased, the rate of ring-flipping is also much reduced. In both cases there are unfavorable steric interactions of the TMP with the nicotinamide ring that need to be overcome for ring-flipping to occur. As one can see from Table 1, the rates of ring-flipping for the binary DHFR·TMP and the ternary DHFR·TMP. PADPR-OCH_3 complexes are fairly similar (less than a factor of 2 different). Since the coenzyme analogue PADPR-OCH_3 has a methoxy group instead of a nicotinamide ring, the observed similar rates indicate that the presence of the nicotinamide ring of the coenzyme is largely responsible for the difference in the rates of ring-flipping between the binary and ternary complex, with the rest of the coenzyme molecule having only a small effect on the TMP flipping. The rate of ring-flipping for form II of the DHFR·TMP·$NADP^+$ (out) is also reduced by only a modest amount as compared to the value in the binary TMP complex. In the $NADP^+$ (out) conformation where the nicotinamide ring is oriented out of its normal binding site and away from the TMP, there must be some accompanying changes in the protein conformation around the binding site that influence the ring-flipping to some extent. These conformational changes would also be consistent with the observed 5-fold cooperative increase in binding trimethoprim in the presence of $NADP^+$ in the form II (out) conformation.

The lack of a simple correlation between the ring-flipping rate and the binding constants is not surprising in that first the interactions involved in forming and dissociating the complex are much more extensive than those directly associated with the trimethoxybenzyl ring, and second, some of the unfavorable steric interactions that need to be overcome to allow ring-flipping might not be part of the association/dissociation pathway. However, it seems reasonable to assume that some of the protein−ligand attractive interactions that would need to be broken to allow ring-flipping would also need to be broken to allow dissociation of the bound trimethoprim. These attractive interactions will have a large contribution from van der Waals interactions involving the hydrophobic groups of the trimethoxybenzyl ring and its protein binding pocket. Some of the entropic factors involved in the ring-flipping might also be influencing the rates of association and dissociation. In an earlier study, it was noted that the rate of ring-flipping is much faster than the rate of dissociation (*5*). If the release of the trimethoxybenzyl ring required for ring-flipping is also part of the dissociation pathway, then it will obviously be only one element in a complicated pathway that would also involve breaking the additional strong interactions involving the diaminopyrimidine ring together with any necessary conformational rearrangements. This view of the dissociation is consistent with a 'zipper' mechanism for binding discussed earlier (*37*). The breaking and reforming of several protein−ligand interactions coupled with the associated conformational changes take place many times within the lifetime of the complex (0.5 s at 298 K) with complete dissociation occurring only when the different dissociation events take place in sufficiently close succession.

The measured thermodynamic parameters of TMP flipping for the ternary complexes DHFR·TMP·NADPH and DHFR·TMP·$NADP^+$ (in) provide some interesting insights. It is seen from Table 1 that, although the ring-flipping rates are fairly similar, form I of the DHFR·TMP·$NADP^+$ (in) complex has much higher enthalpy of activation for this process. However, its entropy term is also considerable larger, and this results in the overall activation energies being similar for the two complexes. The higher enthalpy of activation observed for this complex indicates that during the ring-flipping more electrostatic and hydrophobic interactions need to be broken and/or more unfavorable conformational and steric problems need to be overcome. The measured entropy of activation for DHFR·TMP·$NADP^+$ (in) complex indicates that the ring-flipping process in this case is less ordered than for the NADPH complex. Thus, there are more pathways for negotiating the transition state in the $NADP^+$ (in) complex than in the NADPH complex. In the case of $NADP^+$ (in) complex, the high value of the enthalpy term could result from the steric clashes of the nicotinamide ring with the trimethoprim C′5 methoxy group. There does not seem to be an attractive interaction between the nicotinamide ring and the TMP that would contribute to the enthalpy term. However, in the case of DHFR·TMP·NADPH complex, there appears to be an attractive interaction between the reduced nicotinamide ring and TMP. If this interaction is maintained

during some stages of the flipping, it would reduce the entropy by forcing the process to go through a more constrained pathway.

We have seen that measurements of apparently simple ring-flipping rates are in fact reporting on much more complicated motions and conformational rearrangements in the protein−ligand complex. This dynamic information is complementary to the existing knowledge about structure and binding. Although there is no simple correlation between the dynamic information and the binding constants, some of the factors controlling the dynamics will also be important for the determining the association and dissociation rates in the binding process. A better description of the dynamic processes will eventually provide an improved overall understanding of the interactions in a protein−ligand complex.

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