Structure and dynamics in solution of the complex of *Lactobacillus casei* dihydrofolate reductase with the new lipophilic antifolate drug trimetrexate

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Abstract

We have determined the three-dimensional solution structure of the complex of *Lactobacillus casei* dihydrofolate reductase and the anticancer drug trimetrexate. Two thousand seventy distance, 345 dihedral angle, and 144 hydrogen bond restraints were obtained from analysis of multidimensional NMR spectra recorded for complexes containing 15N-labeled protein. Simulated annealing calculations produced a family of 22 structures fully consistent with the constraints. Several intermolecular protein-ligand NOEs were obtained by using a novel approach monitoring temperature effects of NOE signals resulting from dynamic processes in the bound ligand. At low temperature (5°C) the trimethoxy ring of bound trimetrexate is flipping sufficiently slowly to give narrow signals in slow exchange, which give good NOE cross peaks. At higher temperature these broaden and their NOE cross peaks disappear thus allowing the signals in the lower-temperature spectrum to be identified as NOEs involving ligand protons. The binding site for trimetrexate is well defined and this was compared with the binding sites in related complexes formed with methotrexate and trimethoprim. No major conformational differences were detected between the different complexes. The 2,4-diaminopyrimidine-containing moieties in the three drugs bind essentially in the same binding pocket and the remaining parts of their molecules adapt their conformations such that they can make effective van der Waals interactions with essentially the same set of hydrophobic amino acids, the side-chain orientations and local conformations of which are not greatly changed in the different complexes (similar χ1 and χ2 values).

Keywords: dihydrofolate reductase; dynamics; 15N relaxation; NMR; protein-ligand interactions; ring flipping; structure determination; trimetrexate

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate using NADPH as co-enzyme. This reaction maintains the intracellular pool of tetrahydrofolate required by several folate-dependent enzyme systems involved in important steps in DNA synthesis, such as the biosynthesis of purines and the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate (Blakley, 1985). The important cellular role of dihydrofolate reductase has led to it being used as the target for several clinically useful antifolate drugs. Agents, such as trimethoprim (antibacterial), pyrimethamine (antimalarial), and methotrexate (anticancer), act by inhibiting the enzyme in parasitic or malignant cells. The relatively small size of the enzyme (18–20 kDa) makes it amenable to detailed structural studies (see reviews by Roberts, 1983; Freisheim & Matthews, 1984; Blakley, 1985; Feeney, 1986, 1990, 1996; Feeney & Birdsall, 1993). These have been carried out on complexes of the enzyme using both X-ray (Bolin et al., 1982; Matthews et al., 1985a, 1985b; Sawaya & Kraut, 1997; and references in Gargaro et al., 1998) and NMR methods (Martorell et al., 1994; Morgan et al., 1995; Johnson et al., 1997; Gargaro et al., 1998). These studies have provided information about structures, interactions,
and ionization states in several complexes of dihydrofolate reductase with substrates and inhibitors (Birdsell et al., 1981, 1982, 1984, 1989a, 1989b, 1990, 1997; Coccoli et al., 1981; Gronenborn et al., 1981; Antonjuk et al., 1984; Clore et al., 1984; Bevan et al., 1985; Hammond et al., 1986; Searle et al., 1988; Carr et al., 1991; Cheung et al., 1993; Falzone et al., 1994; Epstein et al., 1995; Polshakov et al., 1995a; Gargaro et al., 1996; Gerothanassis et al., 1996; Nieto et al., 1997).

In the search for improved inhibitors of dihydrofolate reductase, numerous antifolate analogues have been synthesized and investigated (Roth & Cheng, 1982; Baccanari & Kuyper, 1993). One promising antifolate analogue is trimetrexate (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline) (Fig. 1A) (Bertino et al., 1979). Trimetrexate was first prepared by Elslager and co-workers (Elslager et al., 1983) and was investigated initially as a potential antimalarial agent. Subsequently, it was found to have antineoplastic activity in relation to breast, head, and neck cancers (Robert, 1988) and later it was used as an antibacterial in the treatment of Pneumocystis carinii pneumonia (PCP) in AIDS patients (Lin & Bertino, 1987). In 1993 trimetrexate (neutrexin) was approved by the U.S. Food and Drug Administration for the treatment of moderate-to-severe PCP in people with compromised immune systems.

The structure of trimetrexate (Fig. 1A) combines some of the features of trimethoprim (Fig. 1B) and methotrexate (Fig. 1C). Because trimetrexate lacks the terminal glutamic acid moiety of methotrexate, it is more lipophilic and does not need active transport for penetration into the cell (Lin & Bertino, 1991). This significantly increases its efficacy against cells resistant to methotrexate.

Inhibition studies with the human enzyme show that although trimetrexate is about an order of magnitude less potent than methotrexate, it is still an extremely tightly-binding inhibitor (Jackson et al., 1984).

Recent studies on the antipneumocystis activity of trimetrexate have stimulated the synthesis and biological testing of several new nonclassical antifolates (Gangjee et al., 1996). Gangjee and co-workers have used computer-assisted molecular modeling of complexes of dihydrofolate reductase to help in developing novel antifolates (Gangjee et al., 1995). Such rational drug design studies could be improved if more structural information were available for complexes of dihydrofolate reductases and trimetrexate.

In the present work, we report the high resolution solution structure of the binary complex of Lactobacillus casei dihydrofolate reductase with trimetrexate. Detailed information about the conformation of the bound drug and the structure of its binding site has been obtained. This information allows us to compare the binding site with those of methotrexate (Gargaro et al., 1998) and trimethoprim (Martorell et al., 1994) and to examine any differences in their modes of binding. The dynamics of the protein and the bound drug and the specific interactions between the enzyme with the drug have also been characterized.

Results

**Signal assignments for the DHFR-trimetrexate complex**

These assignments for the complex were made by analyzing 2D 1H NMR spectra of the DHFR-trimetrexate complex obtained from COSY, TOCSY, NOESY, and ROEY experiments and 3D 15N-1H NMR spectra from TOCSY-HMQC, NOESY-HMQC, ROEY-HMQC, and HNHB experiments carried out on uniformly 15N-labeled DHFR samples using procedures similar to those described earlier for the DHFR-methotrexate complex (Carr et al., 1991; Gargaro et al., 1998). The 15N-1H HSQC spectrum of the DHFR-trimetrexate complex with all the resonances labeled is shown in Figure 2 and a table containing the 1H and 15N signal assignments is provided in Electronic supplementary material.

In earlier studies on the DHFR-methotrexate complex, almost complete assignments of the proton NMR signals were obtained by analyzing the 3D and 4D spectra from experiments carried out on uniformly 15N and 13C/15N labeled enzyme (Soteriou et al., 1993; Gargaro et al., 1998). These assignments for the DHFR-trimetrexate complex assisted in the assignment of signals from some of the long-chain amino acids in the spectra of the DHFR-trimetrexate complex, because many signals have similar chemical shifts in the two complexes and show similar patterns of NOEs.

Coupling constant and distance-related NMR data were used to derive the stereospecific assignments of β-methylene protons and of valine γ-methyl groups using the program AngleSearch as described in Materials and methods (Polshakov et al., 1995b).

Signals from all the side-chain amide protons of asparagine and glutamine residues could easily be assigned in 2D 13N-1H TOCSY-HMQC and the 3D 13N-1H TOCSY-HMQC and NOESY-HMQC spectra by identifying characteristic NOE patterns involving the NH2 amide side-chain protons. The side-chain amide protons Hγ22 in asparagine and Hδ22 in glutamine were determined as those closer to Hβ protons (or Hγ protons in glutamine) than are Hγ21 or Hδ21, respectively, and the stereospecific assignments of these protons could thus be obtained directly from the intensity information in ROEY spectra. In the 3D ROEY-HMQC spectra, correlations between amide proton of asparagine and glutamine residues sometimes had different polarities for different residues as a result

**Fig. 1.** Molecular structures of (A) trimetrexate, (B) trimethoprim, and (C) methotrexate.
of contributions to the intensities from exchange and NOE effects having opposite signs. Thus, for Q7, Q33, N59, Q92, and N148, strong negative cross peaks between H$_d$$_{21}$ and H$_d$$_{22}$ ~H$_g$$_{21}$ and H$_g$$_{22}$ for asparagine indicated that the NOE contribution is dominant for these protons. In contrast, for other residues, cross peaks with positive polarity were observed and these indicated the predominant occurrence of magnetization transfer as a result of exchange from rotation about the amide bond. For the former residues, the absence of a large exchange contribution indicates hindered rotation about their amide bonds and these residues were subsequently found to be buried inside the protein or involved in hydrogen bond formation. In contrast, the side chains of the remaining asparagine and glutamine residues were found on the surface of the protein.

At 288 K, the H$_e$ signals for all eight arginine residues in _L. casei_ DHFR could be observed in the 3D $^{15}$N-NOESY-HMQC and TOCSY-HMQC spectra. These signals have cross peaks to arginine side-chain H$_d$ protons in the TOCSY spectra. Such signals were subsequently found to be buried inside the protein or involved in hydrogen bond formation. In contrast, the side chains of the remaining asparagine and glutamine residues were found on the surface of the protein.

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**Assignment of ligand signals**

The signals in the $^1$H spectrum of uncomplexed trimetrexate in H$_2$O solution were assigned on the basis of their characteristic chemical shifts, intensities, multiplicities, and intramolecular NOEs (see Table 2). The $^1$H chemical shifts were measured as a function of pH (over the range 4.8 to 9.3), confirming that the site of protonation on the quinazoline ring of trimetrexate is at N1 with a pK$_a$ of 7.93 $\pm$ 0.03. In the $^1$H spectrum of the complex recorded in H$_2$O at 288 K, a signal detected at 15.46 ppm could be assigned to the HN1 proton of the quinazoline ring of protonated bound trimetrexate on the basis of the build-up of large intramolecular NOEs to the HN21 and H8 and smaller NOEs to the HN22 and H7 protons. Similar low field signals have been observed for the HN1 protons of bound methotrexate (16.85 ppm) (Gargaro et al., 1998) and trimethoprim (14.9 ppm) (Martorell et al., 1994). Correlations between H7 and H8 and between HN21 and H8 were detected in the COSY spectrum, thus confirming these assignments. Other signals in bound trimetrexate were assigned on the basis of their intramolecular NOEs, and COSY and TOCSY connections.

The assignments of the CH$_3$-3',5' and H-2',6' protons from the trimethoxybenzyl ring of bound trimetrexate were made by noting...
variations in linewidth resulting from changes in rates of ring flipping with temperature in their 2D and 1D spectra measured as a function of temperature. Part of the $^1$H ROESY spectrum recorded at 288 K showing the exchange cross peaks (in boxes) connecting the exchanging H2’ and H6’, and CH3-3’ and CH3-5’ protons is presented in Figure 3A. The exchange is caused by ring flipping and the signals are readily detectable in the ROESY spectrum because they have opposite signs to other cross peaks that arise from dipolar relaxation interactions. In the $^1$H NOESY spectrum recorded at low temperature (278 K) shown in Figure 3B, the exchange cross peaks between the H2’ and H6’ protons are still detected although with lower intensity. Examination of Figure 3B indicates that it is relatively easy to detect the additional NOE cross peaks that appear when the lines become narrower on lowering the temperature where the slower ring-flipping results in the signals from the ring protons being in slow exchange. This proved to be a very effective method for identifying both inter- and intramolecular NOEs involving the exchanging protons (see below).

The AngleSearch program was used to determine the stereospecific assignments of methylene protons at position 9 in trimetrexate. Intramolecular ROESY intensities were used in the AngleSearch calculations. Because there is no chiral center in the trimetrexate molecule, the stereospecific assignments of the CH2-9 protons in bound trimetrexate have been made in a manner similar to that described for glycine residues in the methotrexate-DHFR complex (Polshakov et al., 1995b). For the two alternative assignments of the methylene CH2-9 protons, ranges of $\tau_1$ and $\tau_2$ torsion angles were obtained from AngleSearch calculations and the correct assignment selected by comparison of these angles with the torsion angles found in the families of NMR structures calculated without using restraints on these torsion angles. This led to the determination of both the stereospecific assignments of the methylene CH2-9 protons and the allowed ranges of torsion angles $\tau_1$ (C5-C6-C9-N10) and $\tau_2$ (C6-C9-N10-C1’) in bound trimetrexate. These constraints were used in the final stage of structure calculations (170° ± 30° for $\tau_1$ and 90° ± 30° for $\tau_2$).

Assignment of protein-ligand NOEs

Once the assignment of the protein and ligand signals had been completed, most protein-ligand NOEs (Fig. 4) could be assigned.
from 2D NOESY spectra recorded with 50 and 100 ms mixing
time at 308, 288 and 278 K.

In most cases ligand NOE partners could be assigned unambiguously
because of their unique chemical shifts or characteristic line
shapes. Signals from the 2',6'-ring protons and the 3',5'-methoxy
protons of the trimethoxyphenyl ring could be uniquely identified
because of their changes in line shape with temperature: decrease
of temperature caused these signals to become sharper due to
slower ring flipping whereas most of the protein signals became
broader. This ability to manipulate the detection of NOEs by rais-
ing or lowering the temperature greatly facilitated the detection of
the protein-ligand NOEs. Furthermore, the availability of NOEs
involving resolved protons from opposite sides of the ring in-
creases the total number of detected NOEs. The method has gen-
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process that can influence the line width of the ligand signals
differently as the temperature is changed. In some cases, protein
partners in protein-ligand NOEs were also assigned by identifying
their characteristic set of NOEs (for example, the trimetrexate H8
proton has NOEs to several protons of L27—Ha, Hz, H81, and H82). For assignment of the remaining NOEs, the ambiguities
were resolved in an iterative manner during the refinement stage of
the structure calculation. A summary of the extensive number of
intermolecular NOEs between trimetrexate and protein protons is
given in Figure 4.

Structure of the DHFR-trimetrexate complex

The structures shown in Figure 5 are from the final 22 calculated
structures superimposed on the backbone of the representative struc-
ture S<sub>ref</sub>. Figure 5A shows a stereoview of the protein backbone
and ligand heavy atoms for all 22 structures. Figure 5B shows
some of the important protein side chains and the ligand in its
binding site. The structures of bound trimetrexate are shown in
Figure 5C. All the structures shown in Figures 5A–C are presented
in the same orientation. Some indication of the precision of the
structures can be realized by noting how well the different calculat-
ed structures are superimposed (pairwise RMSD is 0.5 Å for
backbone atoms and 1.0 Å for all heavy atoms). The absence of
dihedral angle and NOE violations and the small energies for the
experimental constraints (Table 1) provide further evidence for
the precision of the structures. From an examination of the struc-
ture (Fig. 5A) and the PROCHECK analysis (Laskowski et al.,
1993, 1996) of the data, the general features of the structure are
seen to be essentially identical to the overall structural features
found in the solution structure of DHFR-methotrexate (Gargaro
et al., 1998) and in the crystal structures of DHFR-methotrexate.

Backbone dynamics of the DHFR-trimetrexate complex

The overall rotational correlation time τ<sub>c</sub> for the complex of DHFR
with trimetrexate was calculated from the 15N T<sub>1</sub> and T<sub>2</sub> data using
procedures developed by Kay et al. (1989). T<sub>1</sub> and T<sub>2</sub> values were
obtained from a total of 128 residues. Local values of the overall
correlation time were calculated from the T<sub>1</sub>/T<sub>2</sub> ratios of 54 of the
residues (see Materials and methods); the average of these values
gives an overall correlation time of 8.75 ± 0.1 ns at 308 K.

The experimental values of the relaxation parameters were in-
terpreted using the model-free formalism (Lipari & Szabo, 1982),
with extensions to include slower internal motions (Clore et al.,
1990b) and chemical exchange contributions R<sub>ex</sub> to the transverse
relaxation (Clore et al., 1990a) (see Electronic supplementary
material).

Values of the model-free parameters S<sup>2</sup>, τ<sub>c</sub>, and R<sub>ex</sub> have been
obtained for all 128 residues for which experimental relaxation
data were obtained.

Figure 6 shows the measured values of the relaxation parameters
R<sub>1</sub>, R<sub>2</sub>, and NOE (R<sub>N</sub> = T<sub>1</sub>/T<sub>2</sub>) and the calculated generalized order
parameters, S<sup>2</sup>, plotted against the corresponding residue number.
The τ<sub>c</sub> and R<sub>ex</sub> values are given in Electronic supplementary
material.

Ring flipping in bound trimetrexate

Ring flipping of the 3',4',5'-trimethoxyphenyl ring of bound tri-
etrexate has been studied using 2D ROESY and 1D NMR-1H
spectra of the complex recorded over the temperature range 278 to
313 K. In the 2D ROESY (and 2D NOESY) spectra, one can
observe strong exchange cross peaks between H2' and H6' and
between CH2-3' and CH2-5' (Fig. 3). Decreasing the temperature
reduces the intensities of these cross peaks and at the same time
sharpens the signals. This is typical for slow exchange behavior
where separate signals are detected for the exchanging sites and
the line widths of the signals have contributions from the ex-
change process. This dynamic process has been investigated by
line shape analysis of the H6' signal in the 1D spectrum of the
DHFR-trimetrexate complex. This signal is observed as a well-
resolved signal in a noncrowded region of the 1H spectrum (Fig. 7)
and this facilitates measurement of its lineshape. An approximate
estimate of the nonexchange contribution to the linewidth was
obtained from the mean values of the linewidths of several histo-

\[ \text{Structure of the DHFR-trimetrexate complex} \]

The structures shown in Figure 5 are from the final 22 calculated
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\[ \text{Backbone dynamics of the DHFR-trimetrexate complex} \]

The overall rotational correlation time \( \tau_c \) for the complex of DHFR
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estimate of the nonexchange contribution to the linewidth was
obtained from the mean values of the linewidths of several histo-
The rates of ring flipping obtained from a lineshape analysis ranged from 6 s\(^{-1}\) at 278 K to 90 s\(^{-1}\) at 313 K. These values were used to obtain values of enthalpy and entropy of activation (\(\Delta H^\ddagger = 62 \pm 2\) kJ mol\(^{-1}\) and \(\Delta S^\ddagger = -5 \pm 10\) J mol\(^{-1}\) K\(^{-1}\), respectively).

Discussion

The solution structure of the DHFR-trimetrexate complex

Some indication of the precision of the determined structures is provided by Figure 5, which shows the superposition of the final family of 22 structures, and by the plot of the RMSD values against the residue numbers shown in Figure 8. The precision of the structures compares very favorably with that of the structures reported earlier for the DHFR-methotrexate complex (Gargaro et al., 1998). The latter determination was based on NMR studies of \(^{13}\)C/\(^{15}\)N labeled methotrexate and protein samples. The structure determination for the trimetrexate complex was carried out using unlabeled trimetrexate and only \(^{15}\)N-labeled protein. Notwithstanding this, the number of ligand-protein NOEs assigned for the trimetrexate complex (67) was greater than the number obtained for the methotrexate complex (54). The methyl substituted quinazoline ring of the bound trimetrexate has more protons than
Table 1. NMR restraints and structural statistics for DHFR-trimetrexate complex

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<th>A. Restraints used in the final structure calculation</th>
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<th>B. Constraint violations in the final ensemble of 22 structuresa</th>
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<tr>
<td>Number of NOE constraint violations above 0.1 Å</td>
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<td>Number of dihedral angle violations above 3°</td>
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<td>XPLOR energies (kcal mol⁻¹)b</td>
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<th>C. Deviations from idealized geometry</th>
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<td>Bonds (Å) × 10⁶</td>
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<td>Angles (°)</td>
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<td>Improvers (°)</td>
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<th>D. Structural statistics for the final ensemble of 22 structures</th>
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<td>PROCHECK analysis</td>
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<th>E. Atomic RMS differences for the residues 1–162 (Å)</th>
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<td>Backbone (C, Cα, N) atoms</td>
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<th>a Here and below: mean ± standard deviation.</th>
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<tr>
<td>bThe force constant used to calculate (E_{NOE}) was 50 kcal mol⁻¹ Å⁻². The force constant used to calculate (E_{CDIH}) was 200 kcal mol⁻¹ rad⁻².</td>
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<td>c (\langle S \rangle) is the ensemble of 22 final structures, (S_{rep}) is the representative structure (see text).</td>
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<tr>
<td>dValues obtained from pairwise superimposition within ensemble of 22 final structures.</td>
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<td>eValues measured from superimposition on (S_{rep}).</td>
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The corresponding pteridine ring of methotrexate and these protons provide increased opportunities for measuring protein-ligand NOEs. Additionally, the favorable dynamic behavior of the trimethoxy ring of trimetrexate allows the measurement of unambiguous NOEs from protons on each side of the ring at low temperature (278 K). At this temperature the ring flipping is sufficiently slow to allow the H12, H6 and CH₃-3,CH₃-4,CH₃-5 protons to give separate signals in slow exchange and to allow detection of protein-ligand NOEs in the NOESY spectra. These NOEs can be assigned to protein-ligand NOEs by noting their disappearance on raising the temperature where the increased rate of ring flipping broadens the ligand signals. In contrast, the flipping rates of the p-aminobenzoyl ring of methotrexate causes line broadening of its aromatic proton signals such that no signals could be detected at any of the temperatures studied. The low temperature spectra of the DHFR-trimetrexate complex also allowed us to make specific NOE assignments for the separate protons in the trimetrexate NH₂ groups. A further improvement in the structure determination of the trimetrexate complex resulted from the use of many more NOEs involving stereospecifically assigned protons and more hydrogen bonds than were used in the methotrexate structure calculations. Although 18% fewer intramolecular NOEs were used in the calculations for the trimetrexate complex (resulting from the absence of some Hβs/Hβs, Hys/Hβs assignments, which could only be obtained from 13C data), the compensating factors mentioned above result in the determined structures of the two complexes being of similar precision. The availability of the spectral and structural data for the methotrexate complex assisted in the structural determination of the trimetrexate complex. High precision structures for proteins of this size can thus be obtained without using 13C-labeled protein if one has a reference structure to assist in obtaining the initial set of NOE values (see Material and methods).

Comparisons with structures of related DHFR complexes

Figure 9C shows the structures of the three bound inhibitors (Fig. 1A–C) obtained by superimposing the backbone atoms of the representative structures (\(S_{rep}\)) of their DHFR complexes (Kine-
mages 1 and 2 in Electronic supplementary material). It is seen that the inhibitors occupy approximately the same space within the protein and consequently interact with many of the same residues. Although the 2,4-diaminopyrimidine-containing rings occupy essentially the same binding site, the planes of the rings appear to be tilted by 20° with respect to each other (with the trimetrexate structure being in the middle). The trimethoxy benzene ring of bound trimetrexate is perpendicular to that of trimethoprim.

The binding site residues in complexes of dihydrofolate reductase with methotrexate and trimethoprim have been well described in the literature (Bolin et al., 1982; Matthews et al., 1985a; Martorell et al., 1994; Gargaro et al., 1998). In each case, the 2,4-diaminopyrimidine ring moiety binds in a very negatively charged pocket involving the side-chain carboxylate group of D26, the hydroxyl group of T116, and the backbone carbonyl groups of L4 and A97. In the methotrexate complex, there are additional electrostatic interactions involving the carboxylate groups of the methotrexate glutamate side chain with H28 and R57. The "middle" part of the methotrexate molecule binds in a pocket containing several hydrophobic amino acids (Fig. 9A) and many of the same residues are involved in binding the trimethoxy ring of trimetho-

**Fig. 6.** Plots of relaxation data and parameters as a function of residue number. These are shown for the $^{15}$N $R_1$, $R_2$, and $^{15}$N$^1$$H$NOE values measured at 600 MHz and 308 K, and the generalized order parameters $S^2$.

**Fig. 7.** A: $^1$$H$ spectra at 600 MHz of the DHFR-trimetrexate complex (3 mM; D$_2$O buffer) recorded at different temperatures and showing the H-6′ signal of trimetrexate involved in slow exchange with the H-2′ proton due to ring flipping. B: Calculated line shapes for the H-6′ signal. This signal is in a noncrowded region of the observed spectrum, which facilitates the line-shape analysis.
The structure of the DHFR-trimetrexate complex reported here allows us to compare its protein-ligand interactions with those found in the methotrexate and trimethoprim complexes examined previously (Martorell et al., 1994; Gargaro et al., 1998). These comparisons need to be made with caution because not all parts of the structure are equally well defined. However, Figure 8A shows that the regions of the protein with the smallest and largest RMSD values for the DHFR-trimetrexate complex are very similar to those previously reported for the DHFR-methotrexate structure (see Fig. 5 of Gargaro et al., 1998).

Comparison of DHFR-trimetrexate and DHFR-methotrexate structures

The global structure of the DHFR-trimetrexate complex (see Figs. 5, 8, 9) is found to be very similar to that of the DHFR-methotrexate complex with the small differences between the structures being localized mainly to regions around the binding site. This is seen from examination of the RMSD values resulting from the pairwise global fitting of the backbone atoms (C, Ca, and N) of the two families of structures for the DHFR complexes with trimetrexate and methotrexate (Fig. 8). The residues with the largest backbone displacements are in the regions 6–10, 25–31, 72–73, 114–117, and 124–155. The 6–10, 25–31, and 114–117 regions can obviously be implicated in differences between the ligand binding sites as can the 150–155 region, which is on the adjacent β-strand to the 6–10 and 114–117 regions. Differences in the 72–73 region may reflect some uncertainties in the determination of the structure of the loop containing these residues.

The 2,4-diaminopyrimidine ring of each inhibitor binds similarly to the enzyme. In each case the protonated N1 binds to the side-chain carboxylate group of D26 (Fig. 5B) and the 2- and 4-amino substituents are positioned to make interactions with T116, L4, and A97 as described earlier (Bolin et al., 1982; Gargaro et al., 1998). The side chains involved in binding to the 2,4-
atoms obtained from the superposition of structures made over the backbone which can be involved in van der Waals interaction with the in-
molecule is binding in the same binding site.

The positions of the side chains of the interacting hydrophobic residues have not changed substantially between the two different complexes. This can be seen by examining Figure 9A and is supported by the observed small values for the RMSD quotients (Fig. 8C) and the fact that all the residues have very similar measured \( \chi_1 \) and \( \chi_2 \) values in both complexes (see Table 3). Some small differences were seen for L27, F49, and L54 with the largest difference being seen for the L27 \( \chi_1 \) value (17°). Thus, the drugs are adapting their bound conformations to interact with the same hydrophobic amino acid side chains, which bind in general with relatively unchanged \( \chi_1/\chi_2 \) conformations. The small changes in the positions of the interacting side chains in the two complexes (Fig. 9A) must be achieved by the sum of many small relative differences in the backbones of the two complexes.

**Comparison of DHFR-trimetrexate and DHFR-trimethoprim complexes**

Although the structures determined for the DHFR-trimethoprim complex (Martorell et al., 1994) are of lower precision than those of the trimetrexate complex useful structural comparisons are still possible.

The structures of the binding sites in the two complexes are shown in Figure 9B. The trimetrexate hydrophobic binding pocket is seen to contain L19, L27, F30, F49, P50, L54, and A97 and that for trimethoprim contains the same residues except for A97. In the trimetrexate complex, the hydrophobic residues L19 and F30 interact with the quinazoline B ring whereas in the trimethoprim complex, where this ring is missing, these residues can interact with the trimethoxy ring. Although the rings are in different positions in the binding sites of the two complexes, in each case, the side chains of L27 and F49 are sufficiently close to interact with the trimethoxy ring and substituents. The side chains of residues P50 and L54 also could provide some additional interactions in the trimetrexate complex but these will be smaller in the trimethoprim complex. A97 is correctly positioned to interact with the C5 methyl group of bound trimetrexate, which has no equivalent in trimethoprim.

As was found in the earlier comparison of the structures of the trimetrexate and methotrexate complexes, the positions and conformations of the bound trimetrexate, which has no equivalent in trimethoprim.

<table>
<thead>
<tr>
<th>( \chi_1 )</th>
<th>( \chi_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMQ-DHFR</td>
<td>MTX-DHFR</td>
</tr>
<tr>
<td>L19</td>
<td>( -76.8 \pm 6.3^a )</td>
</tr>
<tr>
<td>L27</td>
<td>( -63.7 \pm 4.3 )</td>
</tr>
<tr>
<td>F30</td>
<td>( -170.2 \pm 1.1 )</td>
</tr>
<tr>
<td>F49</td>
<td>( -69.3 \pm 7.9 )</td>
</tr>
<tr>
<td>P50</td>
<td>( 12.8 \pm 0.5 )</td>
</tr>
<tr>
<td>L54</td>
<td>( -60.2 \pm 5.4 )</td>
</tr>
</tbody>
</table>

*Mean value \pm standard deviation.*
conformations of the interacting hydrophobic side chains in the protein are not very different. Thus, once again, effective protein–ligand interactions are formed by the drug adopting a bound conformation which takes advantage of the existing conformations of the interacting side chains of the protein.

Relaxation parameters

The measured values of the 15N relaxation parameters ($T_1$, $T_2$, and NOE) and the calculated generalized order parameter ($S^2$) plotted against the corresponding residue number are shown in Figure 6. The protein in the DHFR-trimetrexate complex appears to have no really flexible parts: the only residue showing high flexibility is the C-terminal A162. Thus, the data indicate that DHFR-trimetrexate complex is globular (average $\tau$ value of 8.75 ± 0.1 ns), which is in good agreement with the determined structure. The mean value obtained for $S^2$ is 0.86 and the standard deviation is 0.05. There are several residues with $S^2$ values below the mean value minus one standard deviation, namely, residues 68–71, 102, 110, 122, 131, 133, 145, 146, 155, 161, and 162. Most of these are near to the unoccupied binding site of the coenzyme or are situated in loop regions.

Ring flipping in bound trimetrexate

The nonlinear fitting of the experimental kinetic data for the trimetrexate ring flipping provides the values: $\Delta H^\ddagger = 62.0 ± 2.0$ kJ mol$^{-1}$ and $\Delta S^\ddagger = -5 ± 10$ J mol$^{-1}$ K$^{-1}$. Interestingly, the value of $\Delta S^\ddagger$ is rather close to the values obtained for ring flipping in 3′,5′-difluorotetrahydrofolate bound to L. casei DHFR ($-15$ J mol$^{-1}$ K$^{-1}$ for binary and $-29$ J mol$^{-1}$ K$^{-1}$ for the ternary complex with NADPH) (Cloro et al., 1984). However, the enthalpy value for ring flipping of trimetrexate is much higher than the values found for 3′,5′-difluorotetrahydrofolate ($\Delta H^\ddagger = 48.1$ kJ mol$^{-1}$ for the binary and $41.4$ kJ mol$^{-1}$ for the ternary complex). There are two components in the value of $\Delta H^\ddagger$: the intrinsic barrier to rotation about the N10-C1′ bond and an additional barrier due to interactions of the benzoyl ring with protein residues including any conformational rearrangements required to relieve steric interactions hindering the ring flipping. For trimetrexate, the intrinsic barrier to rotation would be expected to be less than that for methotrexate, where the intrinsic barrier includes the effects of hindered rotation about the amide bond to the glutamate moiety. Therefore, a substantial contribution to the value of $\Delta H^\ddagger$ (62 kJ mol$^{-1}$) in the trimetrexate complex arises from the additional barrier. Because the structures of the ligand binding sites are fairly similar in the complexes formed with each of the drugs, the difference in $\Delta H^\ddagger$ values for complexes of DHFR with trimetrexate and with 3′,5′-difluorotetrahydrofolate probably reflects additional hydrophobic interactions of the 3′,4′,5′-trimethoxyphenyl ring of trimetrexate with residues in the active site. These interactions would need to be broken before ring flipping can take place, thus contributing to the activation barrier (estimated contribution of 10 to 15 kJ mol$^{-1}$, that is, the difference 62 – 48 kJ mol$^{-1}$).

Materials and methods

Sample preparation

L. casei dihydrofolate reductase was prepared as described previously from an Escherichia coli strain in which the structural gene for the L. casei enzyme had been cloned (Dann et al., 1976; Andrews et al., 1983). Trimetrexate was a gift from Warner-Lambert/Parke-Davis (Pontypool, Gwent). Just over one equivalent of trimetrexate was added as a solid to the solution of dihydrofolate reductase; after 3–10 h standing, undissolved trimetrexate was separated by centrifugation and the final solution dialyzed to remove uncomplexed trimetrexate.

Binding data

Methotrexate and trimetrexate bind very tightly to DHFR, which makes direct measurement of the binding constant difficult. However, the ratios of the binding constants $K_{MTX/DHFR}^0$ and $K_{TMQ/DHFR}^0$ can be estimated from competition experiments. A sample containing a 1:1:1 mixture of DHFR, MTX, and TMQ was made at pH 6.5 in 500 mM KCl and 50 mM potassium phosphate and examined after 2 and 7 days to confirm that complete equilibrium had been reached. At equilibrium, the ratio $K_{MTX/DHFR}^0/K_{TMQ/DHFR}^0$ equals $[\text{DHFR-MTX}]/[\text{DHFR-TMQ}].([\text{TMQ}]/[\text{MTX}])$. Defining $\eta$ as the ratio $[\text{DHFR-MTX}]/[\text{DHFR-TMQ}]$, and assuming that $[\text{DHFR}_{eq}] \sim 0$, which is a good assumption for tightly-binding ligands, it can be shown that for 1:1:1 mixture of DHFR, MTX, and TMQ, ratio $[\text{TMQ}]/[\text{MTX}]$ can be approximated to $\eta$, and therefore $K_{MTX/DHFR}^0/K_{TMQ/DHFR}^0 = \eta^2$. The ratio $\eta$ was obtained from the integrated areas of well-resolved complex-specific signals in the 1D 1H spectra of the complexes. The ratio of binding constants $K_{MTX/DHFR}^0/K_{TMQ/DHFR}^0$ is 20 ± 10. A similar experiment carried out on a sample containing a 1:1:1 mixture of DHFR, TMP, and TMQ gave the ratio of binding constants, $K_{TMQ/DHFR}^0/K_{TMP/DHFR}^0 = 10 \pm 5$. $K_{TMQ}^0$ can then be estimated as $\sim 2 \times 10^8$ M$^{-1}$ using the previously reported values for $K_{MTX}^0$ (Birdsall et al., 1980).

NMR experiments

The NMR experiments were carried out on Varian UNITY and UNITY plus spectrometers operating at proton frequencies of 600 and 500 MHz, respectively. The spectra were recorded at 278, 288, or 308 K on either unlabeled or 15N-labeled protein samples with volumes of 0.6 mL and at concentrations 1 or 4 mM in 500 mM potassium chloride and 50 mM potassium phosphate buffer, pH 6.5, made up either in D$_2$O or in 90% H$_2$O/10% D$_2$O (pH$^\circ$ values are pH meter readings uncorrected for deuterium isotope effects).

Full details of the NMR experiments are provided in Electronic supplementary material. 2D DQF-COSY, TOCSY, NOESY, and ROESY spectra were recorded at temperatures between 278 and 308 K for both H$_2$O and D$_2$O samples of DHFR-trimetrexate. 2D and 3D experiments using uniformly 15N labeled protein, HSQC, TOCSY-HMQC, NOESY-HMQC, ROESY-HMQC, and HNHB were also carried out. Longitudinal and transverse 15N relaxation times ($T_1$ and $T_2$) and 15N[1H]NOE values were measured at 308 K using the pulse sequences described by Kay and co-workers (1992). 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), relative to which the chemical shift of dioxane is 3.75 ppm. The 15N chemical shifts were referenced indirectly to liquid NH$_3$ using the $\gamma$ ratios method (Live et al., 1984; Wishart et al., 1996).
NMR determined constraints

Distance constraints

Interproton distance constraints were obtained from NOEs measured in 2D and 3D NOESY and ROESY spectra recorded in D$_2$O and H$_2$O. The set of distance constraints used for generation of initial structures contained unambiguously assigned NOEs, and also a limited set of NOEs for which the ambiguity was resolved using the NMR structures of the DHFR-MTX complex (Gargaro et al., 1998). The total number of NOEs used for initial structure generation was about 900. When the initial structures were being generated, assignments of additional NOEs were carried out in the usual iterative manner. Cross-peak intensities were classified as strong (0.0–2.5 Å), medium (0.0–3.5 Å), weak (0.0–4.5 Å), and very weak (0.0–5.5 Å). Pseudotetramer center averaging was applied for methyl groups and all distance constraints based on NOEs involving methyl groups had their upper limits increased by 1 Å. An r$^{-6}$ sum averaging, where the r$^{-6}$ distance was weighted by the number of ambiguous NOEs (Nilges, 1995), was applied for all nonstereospecifically assigned pairs of methyl groups, all nonstereospecifically assigned protons in methylene groups and for the H$\beta$ and H$\epsilon$ protons in phenylalanine and tyrosine rings.

The number and distribution of NOEs used in the structural calculations are given in Table 1 (and illustrated in Fig. 2 of Electronic supplementary material). The final restraint list contained 2,070 NOEs. No intraside NOEs between atoms separated by three bonds (e.g., HN=H$, N=H\beta$) were included in the X-PLOR calculations because these (about 430 NOEs) had already been used for defining the torsion angles in the AngleSearch calculations.

Torsion angle constraints and stereospecific assignments

The program AngleSearch (Polshakov et al., 1995b) was used to determine the $\phi$, $\psi$, $\chi_1$, and $\chi_2$ torsional angle constraints and the stereospecific assignments for all the $\beta$-methylene protons and for valine $\gamma$-methyl groups as described previously for the DHFR-methotrexate complex (Gargaro et al., 1998). The error limits obtained from AngleSearch for the dihedral angles were increased to $\pm 30^\circ$ for $\phi$, $\pm 40^\circ$ for $\psi$, and $\pm 30^\circ$ and $\pm 40^\circ$ for $\chi_1$ and $\chi_2$, respectively, to take into account local mobilities and the uncertainties in the Karplus equations used in these studies. The ranges for the trimetrexate torsion angles $\tau_1$ (C5-C6-C9-N10) and $\tau_2$ (C6-C9-N10-C1') were determined from the AngleSearch analysis of ROESY effects between protons of the drug. The final restraint list contained 345 dihedral angles ($\phi$, 123; $\psi$, 120; $\chi_1$, 96; $\chi_2$, 4; trimetrexate, 2).

Hydrogen bond constraints

Hydrogen bond constraints involving backbone NH protons were included in the calculation only at the last stage of structure refinement after they had been identified in the family of structures obtained without their use. Donors of hydrogen bonds corresponding to slowly exchanging NH protons were identified from analysis of the rates of H $\rightarrow$ D exchange for backbone amide protons (measured at 288 K from a series of HSQC spectra of DHFR-trimetrexate complexes recorded between 5 and 880 min after dissolving the sample in D$_2$O as described in Electronic supplementary material). Acceptors of hydrogen bonds were selected if there were only one possible oxygen atom within 3 Å distance of the corresponding amide proton in the family of structures and each hydrogen bond was described with two restraints. The final calculations included 142 hydrogen bond restraints involving backbone NH protons (corresponding to 71 hydrogen bonds).

A hydrogen bond restraint corresponding to the T34 $\gamma$-hydroxyl proton interacting with the carbonyl oxygen of F30 was included in the structure calculation. A signal for the T34 $\gamma$-hydroxyl proton was observed in all NMR spectra recorded in H$_2$O and was also observed for the DHFR-methotrexate complex (Gargaro et al., 1998).

To specify the geometry of the interactions of the trimetrexate HN1 and HN21 protons with the D26 $\beta$-carboxylate oxygens (Polshakov et al., 1995a), two distance restraints and four additional torsion angles were included in the final stage of structure calculation to maintain the planarity of the 2-NH$_2$ group and the pyrimidine ring.

The final constraint list contained a total of 144 hydrogen bond constraints.

Structure calculations

The structure calculations were performed in an iterative fashion using X-PLOR 3.1 (Brünger, 1992). Initially, a set of 100 structures was generated using a combined distance geometry/simulated annealing approach (Nilges et al., 1988, 1991). The distance geometry used subembedding based on the Co, Hα, N, HN, O, C, and Cβ atoms. The ligand was included from the beginning of the calculations. Structures produced by this method were then regularized using simulated annealing consisting of 6 ps of dynamics at 2,000 K with the force constants determining covalent geometry scaled during this stage to their final values, followed by cooling to 100 K with the vdW repulsive term increased from 0.003 to 0.4 and the vdW radii decreased from 0.9 to 0.8 of their standard values and then subjected to 1,000 steps of conjugate gradient minimization. Fifty-four of the structures with essentially the correct fold as judged by comparison with NMR structures of the DHFR-methotrexate complex (Gargaro et al., 1998) were accepted for further refinement. These structures were used to resolve ambiguities in the NOE data set and then subjected to a slow cooling simulated annealing refinement (from 1000 to 100 K during a 20 ps period). This stage of the process was repeated several times including progressively more NOEs and dihedral angle constraints. Twenty-four structures with no NOE violations greater than 0.2 Å and no dihedral angle violations greater than 5° were accepted for the final refinement. At this stage, the hydrogen bonds and the constraints modeling specific interactions between 2,4-diaminopyrimidyl moiety of trimetrexate and the carboxy group of D26 were introduced. The final refinement protocol was essentially the same as the previous one followed by 1,000 steps of conjugate gradient minimization and yielded 22 structures with no distance violation greater than 0.1 Å and no dihedral angle violations greater than 3°. Calculations of the final family of 22 structures (shown in Fig. 5) were carried out with double the earlier cooling MD period (40 ps). Figure 8A shows the RMSD values calculated for the backbone atoms (Ca, C, N) using pairwise global fitting of these 22 structures. Bond lengths were constrained using SHAKE (Ryckaert et al., 1977). Parameter and topology files for trimetrexate were constructed using coordinates from the crystal structure of trimetrexate (Hempel & Camerman, 1988).

Calculations were performed on Silicon Graphics (Power Indigo2) and Sun (Sparc 10) computers. Coordinates for the 22 refined structures have been deposited in the Brookhaven Protein Data Bank.
An initial selection was made of residues that had Tr1bfmr. Structures were visualized using INSIGHT II (Molecular Simulations Inc., Burlington, Massachusetts) and GRASP (Nicholls et al., 1991) and analyzed using X-PLOR 3.1 (Brünger, 1992) and PROCHECK-NMR/AQUA (Laskowski et al., 1993, 1996). The analysis showed that there are no residues in the disallowed regions of the Ramachandran plot (Fig. 3 in Electronic supplementary material). A representative structure S_0 was selected from the ensemble of calculated structures as being the one that is closest to all the other structures and thus gives the lowest sum of pairwise RMSD for the remainder of the structures in the family. RMSD parameters (R_{TMQ}) for both side chain and all heavy atoms of each residue i have been calculated together with their standard deviations. Similar calculations were performed for the family of 21 calculated structures of the complex of MTX (Gargaro et al., 1998). For comparison of solution structures of MTX and TMQ, similar parameters were also obtained using cross-pairwise superimposition of each structure of MTX on each structure of TMQ in their families (462 pairs). For comparison of two families, a parameter \( \rho' = (R_{TMQ/MTX})^2/(R_{TMQ} \cdot R_{MTX}) \) was calculated. This parameter is especially useful when one is comparing results for superimposition on all heavy atoms. For example, residues with ill-defined side-chain conformations have large values of \( R_{TMQ/MTX} \), \( R_{TMQ} \), and \( R_{MTX} \), and the values of \( \rho' \) will be closer to 1. However, residues that have well-defined side chains within families but that have large differences between two complexes will have large values of \( \rho' \) (see Fig. 8). In Figure 8C, only \( \alpha \)-helix B (between residues 26 and 28) shows a significant local displacement between the two DHFR complexes probably due to the interaction between H28 and the glutamate moiety of methotrexate, which is absent in the trimethoprim complex.

**T1, T2 and \(^{15}\text{N}(\^1\text{H})\) NOE data analysis**

The relaxation data were analyzed using the “model-free” approach of Lipari and Szabo (1982) with the assumption of isotropic tumbling. The spectra used in the \( T_1 \), \( T_2 \), and \(^{15}\text{N}(\^1\text{H})\) NOE experiments were processed and analyzed with the FELIX program (Molecular Simulations Inc.). The time domain data were zero-filled to 1K and 2K complex points in the \( t_1 \) and \( t_2 \) dimensions, and a Gaussian window function was applied in both dimensions before transformation. The \( T_1 \) and \( T_2 \) values (giving the rates \( R_1 \) and \( R_2 \), respectively) and their standard deviations were obtained from nonlinear fits of both integral peak volumes and the peak intensities. Standard deviations of \(^{15}\text{N}(\^1\text{H})\) NOE values were calculated using the RMS noise of background regions (Farrow et al., 1994). For most of the residues, the results were identical, but for the residues with signals situated in more crowded regions the results obtained from peak intensities (rather than from volumes) had smaller standard deviations, and therefore only these were used for further analysis (results shown in Fig. 6). \( T_1 \) and \( T_2 \) values were obtained from a total of 128 residues.

The overall correlation time \( \tau_c \) for the complex of DHFR with trimethoprim was calculated from the \( T_1/T_2 \) ratios (Kay et al., 1989). An initial selection was made of residues that had \( T_1 \) and \( T_2 \) values within one standard deviation from the mean values over all residues (93 of 128 residues). Of these, the 54 residues that had \( T_1/T_2 \) ratios within one standard deviation of the mean of the subset of 93 were then selected for further analysis. The calculations yield an average \( \tau_c \) value of 8.75 ± 0.1 ns. The overall correlation time was treated as a fixed parameter in the analysis of the relaxation data in terms of the model-free formalism.

Calculation of the model-free parameters from the measured relaxation rate constants and the NOE enhancements was performed by Powell minimization (Press et al., 1992) of a global target function \( \chi^2 \) summed over all residues using software written in-house (see Electronic supplementary material). In this analysis, \(^{15}\text{N}(\^1\text{H})\) dipolar relaxation and \(^{15}\text{N} \text{CSA} \) relaxation were examined using equations in the literature (Kay et al., 1989; Farrow et al., 1994). Values of 1.02 Å and 160 ppm were assumed for the N-H bond-length and \(^{15}\text{N} \text{CSA} \), respectively. For some residues, an exchange term \( R_{ex} \) was included to represent line broadening due to exchange processes on a timescale much slower than the overall rotational correlation time.

The relaxation data were fitted to three functional forms of the model free spectral density functions given in the literature (Lipari & Szabo, 1982; Clore et al., 1990a, 1990b) and described in Electronic supplementary material.

**Supplementary material in Electronic Appendix**

File: tmqsupp1.doc (The tables are in Word 6.0 (RTF) with Courier font; the text is in Word 6.0 (RTF) Times New Roman font.)

File: Fig_tmq.zip contains Figure1.bmp

File: Fig_tmq.zip contains Figure2.bmp

File: Supplementary

File: Dhfr_tmq.kin contains two kinemage figures

NMR experimental details

Relaxation data analysis and parameters

Table 1. (Supplementary). \(^1\text{H} \) and \(^{15}\text{N} \) chemical shift assignments for the DHFR-trimethoprim complex at 308 K.

Table 2. (Supplementary). NOE, dihedral angle, and hydrogen bond restraints used for the final X-PLOR structure calculations.

Table 3. (Supplementary). Relaxation data and calculated parameters for the complex of DHFR and trimethoprim.

Fig. 1. (Supplementary). Plots of relaxation data and parameters as a function of residue number.

Fig. 2. (Supplementary). Plot of the number and distribution of NOEs against the amino acid sequence.

Fig. 3. (Supplementary). A Ramachandran plot of the torsion angles for all residues of the 22 final structures.

Kinemage 1. Ribbon representation of DHFR in its complex with trimethoprim.

Kinemage 2. Comparison of three representative structures of binary complexes of DHFR-with trimethoprim, methotrexate, and trimethoprim.

**Acknowledgments**

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