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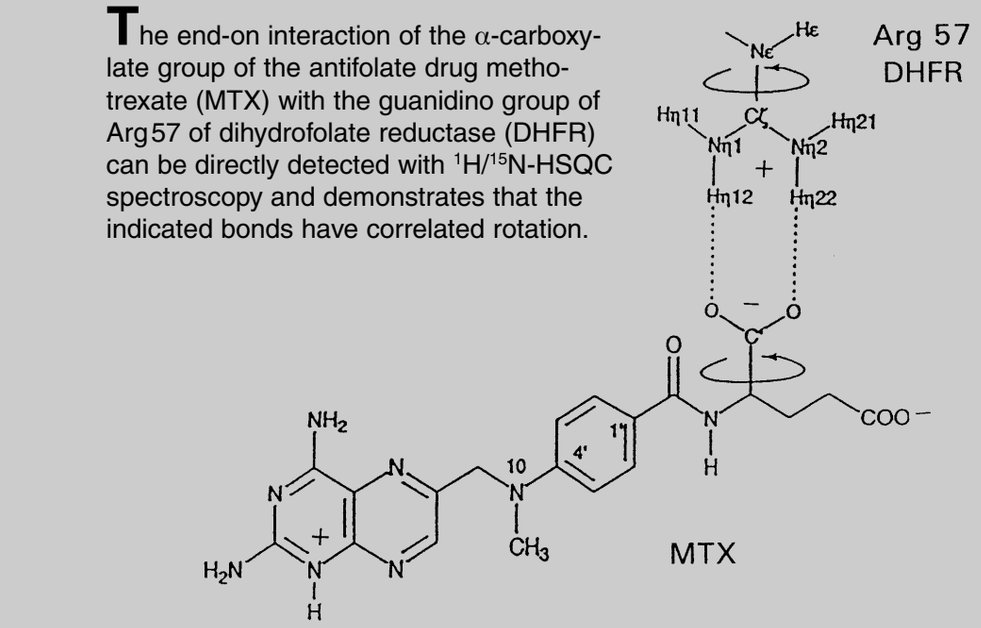
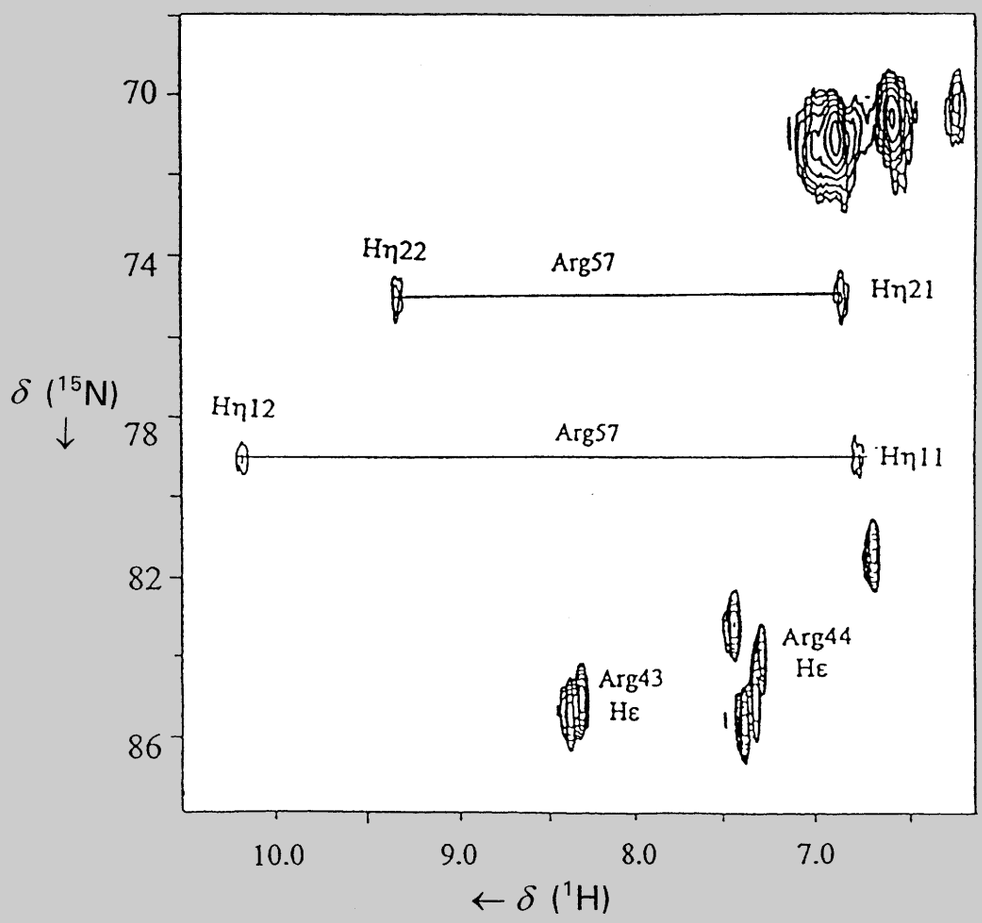
The end-on interaction of the α-carboxylate group of the antifolate drug methotrexate (MTX) with the guanidino group of Arg57 of dihydrofolate reductase (DHFR) can be directly detected with $^{1}H/^{15}N$-HSQC spectroscopy and demonstrates that the indicated bonds have correlated rotation.





NMR Studies of Ligand Binding to Dihydrofolate Reductase

James Feeney*

It is just 50 years since Farber and coworkers reported that remission of some childhood leukaemias could be achieved using the folate antagonist aminopterin. Earlier research in several laboratories had identified the antifolate properties of agents based on the 2,4-diaminopyrimidine structure—pioneering work that eventually led to the development of drugs such as trimethoprim (antibacterial) and pyrimethamine (antimalarial). The medical importance of this work was acknowledged in 1988 when Hitchings and Elion shared the Nobel Prize in Physiology and Medicine (with Black) in recognition of their work on inhibitors of purine and pyrimidine metabolism. The target enzyme for the antifolate drugs is dihydrofolate reductase (DHFR) and its discovery by Futterman in 1957 triggered an avalanche of scientific investigations which has continued unabated to the present time. Complexes of DHFR with small molecules became early paradigms for studying drug–receptor interactions and received the attention not only of molecular pharmacologists and biochemists but also of chemists and physicists attracted by the possibility of carrying out investigations at a molecular level on a well-defined system of medical significance. After the discovery of the enzyme, many DHFRs from various sources were purified and subjected to detailed biochemical and biophysical investigation. More than 60 structures of DHFR complexes have now been determined by X-ray crystallography, many of them in the laboratories of Kraut and Matthews. These structures have allowed detailed discussions of the enzyme mechanism and assisted in the building of structural models of DHFR complexes with novel inhibitors. More recently, NMR spectroscopy has been used to determine the structures of DHFR complexes in solution. The major strengths of the NMR technique have been its unique capabilities for probing specific interactions of enzymes with substrates and substrate analogues, for characterizing the multiple conformations of the complexes, and for measuring rates of a wide range of dynamic processes in these systems. Such studies on complexes of dihydrofolate reductase form the basis of this review.

Keywords: enzymes · NMR spectroscopy · protein dynamics · protein–ligand interactions · protein structures

1. Introduction

Currently there is a great deal of interest in investigating the molecular recognition processes involved in forming drug–receptor complexes. The driving force for such studies is the presumption that a molecular understanding of the origins of binding specificity might eventually provide a reliable basis for rational drug design. One of the most extensively studied systems is the enzyme dihydrofolate reductase (DHFR) and its interactions with antifolate drugs.[1–27] This enzyme catalyzes the reduction of folate **1** and dihydrofolate **2** to tetrahydrofolate **3** [Eqs. (1, 2)].

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$$\text{Folate } (\mathbf{1}) + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{7,8-dihydrofolate } (\mathbf{2}) + \text{NADP}^+ \quad (1)$$

$$\text{7,8-Dihydrofolate } (\mathbf{2}) + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{5,6,7,8-tetrahydrofolate } (\mathbf{3}) + \text{NADP}^+ \quad (2)$$

DHFR is an essential cellular enzyme since the final product of the catalysis, tetrahydrofolate, is an important precursor required in several biosynthetic processes involved in the production of purines, pyrimidines, and some amino acids.[6] DHFR is thus an excellent target for drugs aimed at selectively inhibiting the enzyme in invasive cells. There are several clinically useful antifolate drugs including trimethoprim (**4**, antibacterial), methotrexate (**5**, anticancer), and pyrimethamine (**13**, antimalarial), which act by inhibiting the enzyme in parasitic or malignant cells. Trimethoprim, for example, is 16500 times more effective as an inhibitor of the bacterial enzymes than the mammalian enzymes.[5]

Dihydrofolate reductases are generally small proteins (18000–25000 Da in most species) and have no disulfide bonds nor metal ion requirements. A fascination with the

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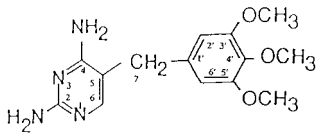

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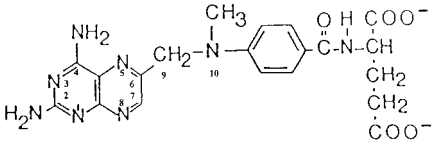

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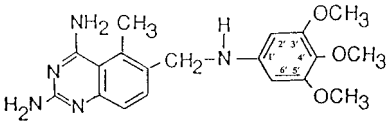
enzyme has dominated the research activities of many scientists and several reviews[5–12] have chronicled the progress in the field. Many modified DHFRs have been prepared by site-directed mutagenesis and have been studied in order to answer specific questions relating to enzyme catalysis or ligand binding.[27–31] Extensive binding studies of inhibitors and coenzymes to DHFR from *Lactobacillus casei* have been carried out and examples of measured association constants (K_a values being measured at pH 6.5 – 7.0) for typical binary complexes are: methotrexate (**5**, $2 \times 10^9\,\text{M}^{-1}$), trimethoprim (**4**, $2 \times 10^7\,\text{M}^{-1}$), trimetrexate (**6**, $\sim 2 \times 10^8\,\text{M}^{-1}$), NADPH ($1 \times 10^8\,\text{M}^{-1}$), and $NADP^+$ ($4.5 \times 10^4\,\text{M}^{-1}$).[32–35]



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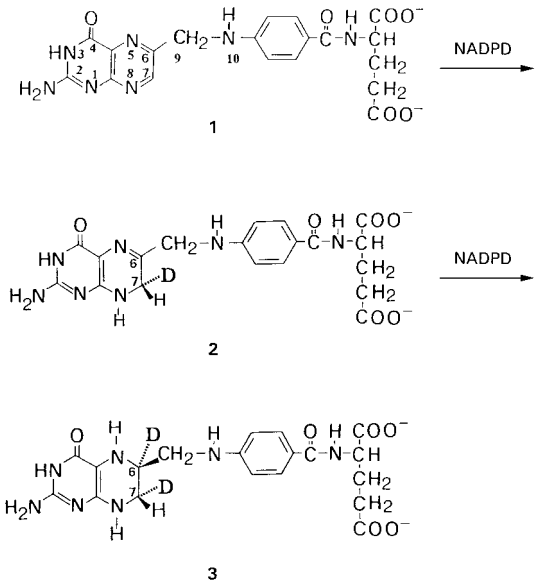
The formation of the ternary complex with NADPH and a substrate analogue is often accompanied by cooperative (positive or negative) effects where the two ligands influence each others binding.[34, 35] These effects can be large and influence the binding by as much as a factor of 2000 in some cases. Differences in the cooperative binding of ligands to enzymes from different species can sometimes contribute to the specificity of binding. For example, the specificity of the binding of trimethoprim to the bacterial enzymes over the mammalian enzymes is partly a result of differences in the cooperativity of the binding with NADPH: the cooperativity factor of 200 observed for the binding to the bacterial enzymes is much reduced in complexes with mammalian enzymes.[33] Since it is likely that the DHFR-NADPH-inhibitor ternary complex is the therapeutically relevant complex, these affects of the coenzyme on the binding specificity will be of considerable significance in drug design. Although DHFR has been studied comprehensively there are still many unanswered questions relating to the origins of the binding specificity and cooperativity.

The structures of many DHFR complexes have been determined by X-ray crystallography[13–16] and such structures have been used to assist in modeling structures of related ligand complexes of DHFR using quantum chemistry calculations.[36, 37] In recent years advances in NMR methods have enabled structures of moderately-large proteins to be determined and structures of several dihydrofolate reductase complexes have now been reported.[17–21] However, the major power of the NMR technique is its ability to provide information that is inaccessible by other methods. For example, NMR spectroscopy has proved to be an excellent method for probing specific protein – ligand interactions, multiple conformations, and dynamic processes in protein – ligand complexes.[22–26]

2. Insights into the Mechanism of Dihydrofolate Reductase

DHFR reduces folate (**1**) first to 7,8-dihydrofolate and then to 5,6,7,8-tetrahydrofolate [Eqs. (1, 2)], with the folate reduction being 100 times slower than the subsequent reduction of 7,8-dihydrofolate in the reaction catalyzed by *L. casei*.[38] The reduction of 7,8-dihydrofolate involves transfer of the 4-pro-*R* hydrogen atom of NADPH to the C6 position of dihydrofolate.[39, 40] We have used NMR methods to determine the stereochemistry of this reduction process. For this, we prepared selectively deuterated 5,6,7,8-tetrahydrofolate by reducing folate with *L. casei* DHFR and NADPH specifically deuterated at its 4-pro-*R* position.

James Feeney was born in 1936 in St. Helens, England. He graduated from the University of Liverpool (BSc, 1958; PhD, 1960) and was awarded a DSc by the university in 1975. From 1960 – 1964 he was a Lecturer in Chemistry at the University of Liverpool. He then joined Varian Associates and subsequently was appointed Director of Varian European Laboratories (1967 – 1969) in Zurich. Since 1969 he has worked with the Medical Research Council, first at the MRC Molecular Pharmacology Unit, Cambridge (1969 – 1972) and then at the National Institute for Medical Research, Mill Hill (1972 – present). Dr. Feeney is Head of the Molecular Structure Division (since 1989) and Controller of the MRC Biomedical NMR Centre (since 1980) at the National Institute for Medical Research. He is a Visiting Professor at the University of Surrey, a Fellow of the Royal Society of Chemistry, and has an Honorary Doctorate from the University of Roskilde, Denmark.



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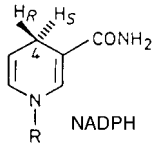
We then determined the sites of deuteration in the product by comparing the ^{1}H spectra of the deuterated and non-deuterated tetrahydrofolates. It could be shown that the deuterons which are transferred to the C6 and C7 positions are both added onto the same face of the pterin ring[41, 42] (see structure **3**). The absolute configuration of the hydrogen atom at the 6-position of tetrahydrofolate was already known to be 6*S* from earlier X-ray crystallographic studies.[43, 44] Thus, the NMR results for the enzymic deuteration of folate gave the configuration of the added proton at the 7-position as 7*S*, that is, on the same face as the one in the 6-position. In the X-ray structure of the complex of DHFR with methotrexate (**5**, MTX) and NADPH,[13, 14] the "transferred" proton 4-H_R of NADPH is located near the opposite face of the MTX pteridine ring to that which receives the protons in the folate reduction.[13, 14, 41–45] Thus, although methotrexate (**5**) and folate (**1**) have similar structures, differing only at positions C4 and N10, the pteridine ring of **5** binds to DHFR in a different orientation to that of **1**[41, 42] and 7,8-dihydrofolate.[13, 43, 45] This behavior has been confirmed in subsequent X-ray[16] and NMR structural studies,[27, 46] where the pterin ring in the DHFR – folate complex was found to be turned over by about 180° relative to its orientation in the DHFR – MTX complex. The finding that such similar molecules can bind in very different ways emphasizes the value of carrying out detailed structural studies in evaluating inhibitor design.



The mechanism of protonation of N8 (for folate) and N5 (for dihydrofolate) as the first stage of each reduction step is still not fully understood. Selinsky and co-workers[47] have examined binary complexes of bovine DHFR with folate and dihydrofolate (labeled with ^{15}N at N5 and ^{13}C at C6) and showed that N5 was not protonated in either of the complexes. Mutagenesis studies modifying Asp27 in *E. coli* DHFR have indirectly implicated this residue in the enzymatic reduction.[31] Asp27 is the only residue with an ionizable group that is close to the active site (equivalent to Asp26 in *L. casei* DHFR and Glu30 in human DHFR). The X-ray structure analysis[16] indicates that this residue is not sufficiently close to either N8 or N5 to be involved in direct protonation. The oxygen atoms of the carboxyl group of the conserved Asp residue are hydrogen bonded to one of the protons in the 2-NH_2 group and to the N3 proton of folate.[48, 49] Protonation schemes for N8 and N5 have been proposed in which the Asp residue promotes the formation of a protonated enolic form of the pteridine ring as an intermediate prior to reduction at N5 (Cannon and co-workers[50] have carried out calculations that suggested that the substrate could form an enol tautomer). However, studies of binary complexes of ^{13}C- and ^{15}N-labeled folates show that bound folate exists in the keto form for human and bacterial enzymes.[46–49] Blakley et al.[49] have also shown that N3 remains protonated even at pH 9.5 in the folate complex with human DHFR. They noted the predominance of the imino – keto form of bound folate and concluded that protonation schemes involving the enolic forms are unlikely to be correct. Blakley and co-workers[49] detected no change in the ionization state of the Glu30 carboxylate group over the pH range 5 to 7 in the binary complex of folate with human DHFR and they concluded that ionization of Glu30 is unlikely to be the origin of the observed pH dependence of hydride transfer (apparent pK ≈ 6.0).

Similar results were found for the equivalent Asp26 in the complex of *L. casei* DHFR with folate.[51] Oefner and co-workers[16] detected a bound water molecule near to N5 in their crystal structure studies of the complex of human DHFR with folate and suggested that this water molecule is a likely candidate for mediating a proton transfer to N5. A similar water molecule has been detected in X-ray[14] and NMR studies[54] of the *L. casei* DHFR-MTX-NADPH complex (water molecule 253 shown in Figure 1) and also in NMR studies of human DHFR complexes.[55] The detection of this long-lived water molecule in solution supports its possible involvement in the proton transfer to N5 of dihydrofolate. A major complication of all these NMR studies is that none of the data has been obtained on the relevant ternary complex containing the substrate and NADPH but rather on related complexes.

Benkovic et al.[28, 29, 56, 57] and Blakley et al.[58] have also carried out extensive kinetic studies on several DHFRs. For example, Andrews, Benkovic, and co-workers[56] have shown that for the *L. casei* enzyme, the dissociation of the product is the rate-limiting step for the steady-state turnover at low pH values. The product, tetrahydrofolate, only dissociates from the enzyme after $NADP^+$ is replaced by NADPH.

3. Signal Assignments and Nuclear Overhauser Effects

3.1. Assignment of the NMR Signals of the Protein

Multidimensional NMR methods[59, 60] have been used in combination with isotopically labeled proteins to obtain complete signal assignments for several complexes of DHFR from bacterial[52, 61–63] and mammalian species.[21, 53] In our own

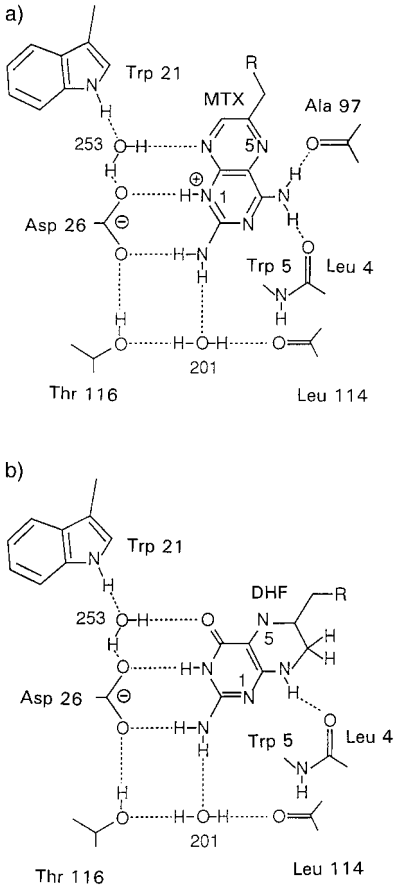
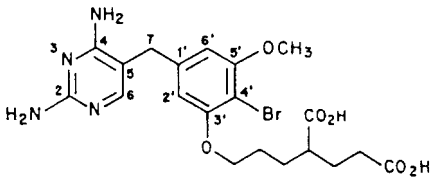


Figure 1. Schematic diagrams showing the location of water molecules 201 and 253 in the a) methotrexate binding site of the *L. casei* DHFR-MTX-NADPH complex as determined by X-ray crystallography[14] and b) proposed dihydrofolate (DHF) binding site (from Bolin et al.);[14] Trp5 has been incorporated into the original figure from Bolin et al.[14] (Reprinted with permission from Bolin et al.[4])



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NMR studies of complexes of *L. casei* DHFR with **4**–**7**, the assignments were obtained[17–20, 61, 62] using samples containing ^{15}N and $^{13}C/^{15}N$ uniformly labeled DHFR and specifically labeled ligands.[61, 62] The availability of over-expressing *E. coli* strains containing the *L. casei* DHFR gene simplifies the preparation of isotopically labeled proteins.[64] The sequential assignments of the backbone resonances were assigned from 3D TOCSY–HMQC, NOESY–HMQC,[65] HMQC-NOESY-HMQC,[66–68] HNCA,[69] HNHA,[70] and HNHB experiments,[71] while 3D $^{13}C/^{1}H$ HCCH–COSY and HCCH–TOCSY[72, 73] experiments enabled most of the side-chain resonances to be assigned for the complex with methotrexate (an example of the latter is shown in Figure 2). Once the detailed assignments had been made for the complex of DHFR with methotrexate, it was relatively easy to transfer the assignments to related complexes because of the similarities in the connectivity patterns seen in the 2D spectra of the different complexes.

In the days before the NMR methods for making sequential assignments were available, assignments were often made by using NOE measurements to correlate and assign resonances from nuclei that were close to each other in the crystal structure of the complex.[76] This method assumes that the solution and crystal structures are similar. Many of the early assignments for complexes of *L. casei* DHFR were made in this way, and they all proved to be in complete agreement with the NMR sequential assignments made subsequently.[61, 62, 76] The assignment method based on crystal structure information is still a useful approach for very large proteins (> 50 kDa) where scalar coupling correlations between nuclei are difficult to detect.

Selective isotopic labeling of DHFR has been used to simplify spectra and assist in spectral assignments. Several selectively deuterated *L. casei* DHFRs were prepared by biosynthetic incorporation of deuterated amino acids; by comparing spectra of the nondeuterated and selectively deuterated samples, it was possible to detect and assign those ^{1}H signals which are present in the former and absent in the latter.[77–82] By examining 2D COSY spectra from DHFR-containing γ-Me-deuterated valine[78, 79] and α-deuterated valine,[80] it was possible to assign the signals for the protons normally at the deuterated sites. We have prepared *L. casei* DHFR containing (2*S*,4*R*)-[5,5,5-$^{2}H_3$]leucine[81] and have made stereospecific assignments for Leu methyl groups by examining the ^{1}H COSY spectra of the deuterated and nondeuterated samples (see Figure 3).

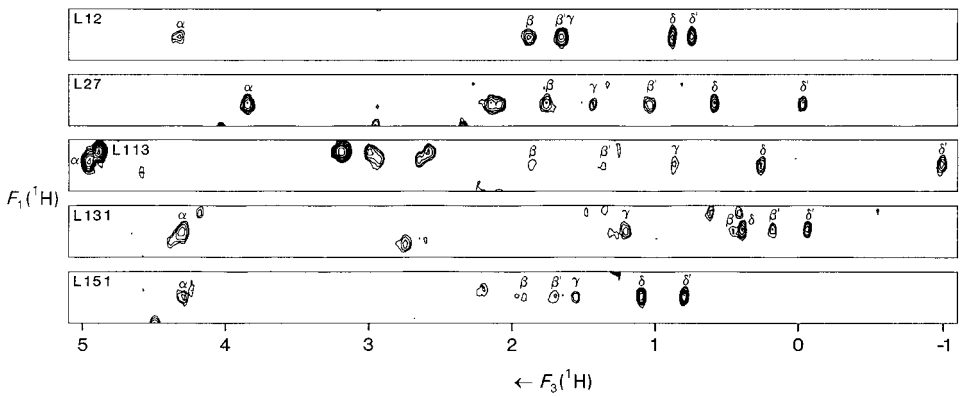


Figure 2. Representative F_1/F_3 strips from the HCCH-TOCSY spectrum of the DHFR–MTX complex taken at the α-carbon shifts (F_2) of five leucine residues. The spectra can be considered as a series of 2D TOCSY[74, 75] slices ($^{1}H(F_1)/^{1}H(F_3)$) at different ^{13}C chemical shifts (F_2). The labeled cross-peaks are typical of the through-bond correlations seen for the α-protons (F_1) of all 13 leucine residues in DHFR. (Reprinted with permission from Soteriou et al.[62])

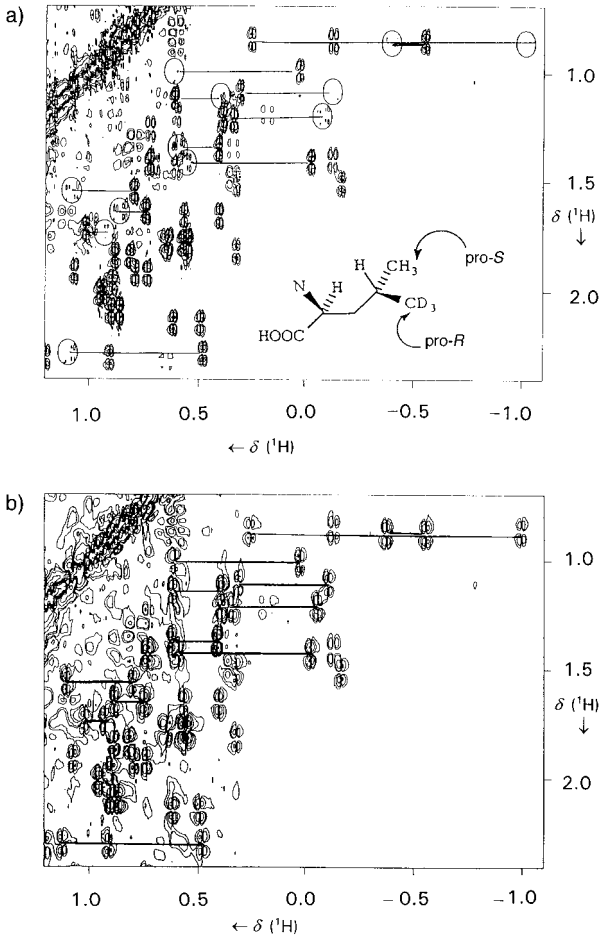


Figure 3. The high-field "aliphatic" region of the 2D DQF-COSY ^{1}H contour plot for the *L. casei* DHFR – MTX complex recorded at 308 K. a) The complex of the selectively deuterated enzyme incorporating (2*S*,4*R*)-[5,5,5 – $^2H_3$]leucine; b) the complex with the nondeuterated enzyme. The positions of the leucine cross-peaks involving the 4-pro-*R* methyl group are circled in (a) and the methyl pairs from each leucine are joined with lines. (Reprinted with permission from Ostler et al.[81])

In some experiments, *L. casei* DHFR samples containing several deuterated amino acids were examined.[79, 82] This is a particularly useful approach for examining aromatic amino acid signals and their NOE connections. The spectrum shown in Figure 4 illustrates the considerable simplification that can be obtained. Spectral simplification can also be achieved by using photochemical CIDNP experiments (CIDNP = chemically induced dynamic nuclear (spin) polarization) experiments to enhance signals from surface-accessible aromatic residues in complexes of DHFR with inhibitors.[83]

Various ^{13}C- and ^{19}F-containing amino acids have been biosynthetically incorporated into the enzyme and NMR measurements then used to study the effects of ligand binding.[84–86] A remarkable result was obtained for *L. casei* DHFR containing 6-F-Trp, where the fluorine substituents in two of the Trp residues (Trp 5 and Trp 133) are in sufficiently close proximity to show a through-space ^{19}F-^{19}F scalar spin – spin coupling.[84]

3.2. Assignment of the NMR Signals of the Ligands

Ligand nuclei are obviously well placed to provide direct information about the binding site in the complex. Assignments of ligand resonances are crucial for detailed structural studies because they are the starting point for collecting the intermolecular protein – ligand NOEs required for determining distance restraints. For weakly binding ligands ($K_a \leq 10^3\,\text{M}^{-1}$) that show fast exchange behavior between bound and free species the chemical shifts of the bound ligand can easily be calculated from the analysis of the binding curves.[87, 88] Very tightly bound ligands ($K_a > 10^7\,\text{M}^{-1}$) show slow exchange behavior and give separate signals for the bound and free ligand. These are often assigned by examining isotopically labeled analogues in combination with various experimental procedures. In the simplest experiments the

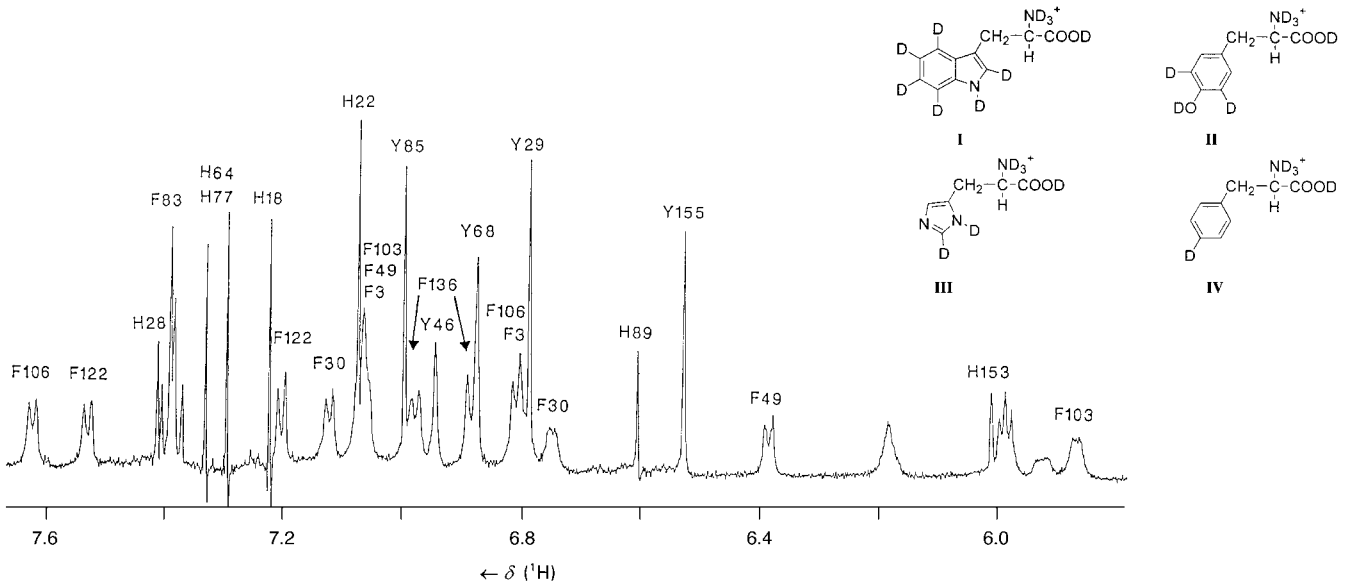


Figure 4. The aromatic region of the resolution-enhanced ^{1}H spectrum of a selectively deuteriated *L. casei* dihydrofolate reductase-methotrexate complex recorded at 600 MHz. The selectively deuterated aromatic amino acids **I** – **IV** were incorporated into the DHFR sample. (Reprinted with permission from Birdsall et al.[82])

complexes formed with the labeled ligands can be examined directly using ^{15}N or ^{13}C NMR spectroscopy: only signals from nuclei at labeled positions are observed and thus the assignment problem is usually trivial. Several studies of this type have been carried out in our laboratory involving isotopically labeled folate,[89, 90] methotrexate (**5**),[91] and trimethoprim (**4**).[92–94] Blakley and co-workers[49] have also used this approach to examine isotopically labeled folate (**1**) bound to human DHFR and Huang and co-workers have likewise studied *E. coli* DHFR complexes containing labeled **1** and **4**.[95, 96] Isotope editing or filtering pulse sequences can also be used. Heteronuclear multiple (and single) quantum coherence (HMQC and HSQC) experiments allow the scalar coupled $^1H/X$ pairs to be detected selectively (each characterized by both the ^1H and $^{13}C/^{15}N$ frequencies, the X nuclei being detected indirectly by the ^1H nucleus).[97, 98]

For complexes formed with less tightly binding ligands ($K_a \approx 10^6\,M^{-1}$), the dissociation rates can be sufficiently fast to allow magnetization transfer experiments to connect the separate resonances seen for the bound and free species. Because the assignments in the free ligand are usually known with certainty, the assignments of the connected signals from the bound species can then be made. We have used transfer of magnetization methods to assign signals from $NADP^+$,[100] trimethoprim (**4**),[101] folate (**1**),[32, 101] and pyrimethamine[102] analogues bound to *L. casei* DHFR. During the course of these studies the first transferred NOE experiments for determining intramolecular NOEs in bound ligands were carried out.[103, 104] In this method cross relaxation (NOE) between two protons in the bound ligand is transferred to the free molecule by exchange between bound and free species. The negative NOEs from the bound state can thus be detected from experiments on the signals of the free ligand in slow exchange with bound species (Figure 5) or on the averaged signals for free and bound ligands when in fast exchange. These NOEs can then used to determine the conformation of the bound ligand.[103] Quantitative results are best obtained for systems in fast exchange where the exchange rates are much faster than the relaxation rate of the free proton.[105]

DHFR complexes formed with ligands containing isotopes such as ^3H, ^{19}F, and ^{31}P have also been examined.[102, 106–110] For example, ^{19}F NMR has been used to examine complexes of *L. casei* DHFR formed with fluorine-containing pyrimethamines; their simple ^{19}F spectra are ideal for monitoring multiple conformations and dynamic processes in the complexes (see Section 8 on multiple conformations).[102, 107, 110]

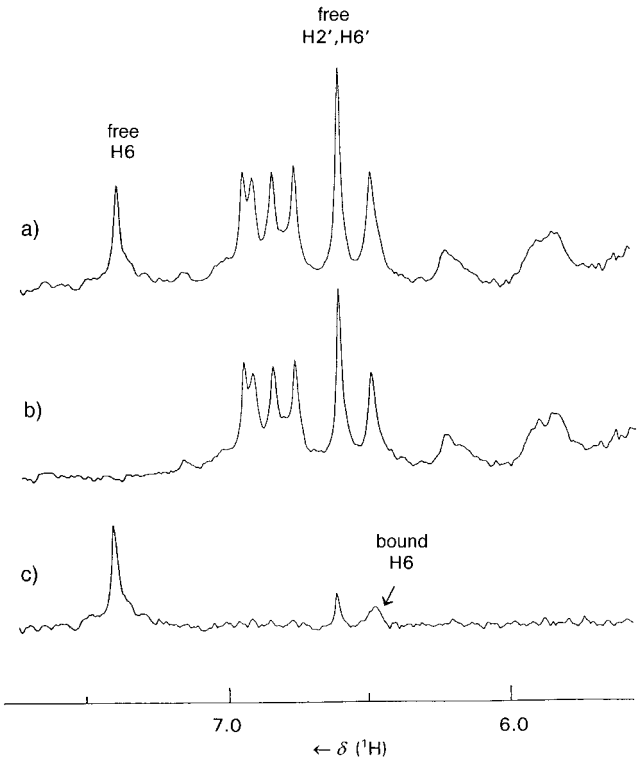


Figure 5. The aromatic region of the 270 MHz ^1H spectrum at 319 K of a selectively deuterated DHFR in the presence of 2.7 equivalents of trimethoprim (**4**). a) Control spectrum without irradiation. b) Spectrum after irradiation at the H6 proton frequency of free trimethoprim. c) Difference spectrum showing a negative NOE to the free H2′,H6′ signal (at $\delta = 6.61$) and a transfer of magnetization to the bound H6 signal (at $\delta \sim 6.5$). Transferred NOEs[103, 104] are observed between the H6 and the H2′,H6′ protons of free trimethoprim in the presence of trimethoprim bound to *L. casei* DHFR: the negative NOEs observed in (b) and (c) on the free H2′,H6′ signal on irradiation of the free H6 signal are transferred by an exchange of magnetization between bound and free trimethoprim and reflect the negative NOEs between these protons in the bound species. (Reprinted with permission from Cayley et al.[104])

3.3. Ligand – Protein NOE Measurements

Isotope editing experiments, as pioneered by Fesik and co-workers[97] as well as Otting and Wüthrich,[146] can be used to assign protein – ligand NOEs. For example, 2D HMQC – NOESY experiments can be used for measuring NOEs from the isotopically labeled ligand to the protein. The 3D NOESY – HMQC experiment is a powerful extension of this approach that allows selective detection of NOEs between ligand protons (attached to ^{13}C or ^{15}N) and neighboring protons in the protein, with the observed NOESY cross-peaks being dispersed over the chemical shift of the X nucleus. This method considerably simplifies the NOESY spectrum at any particular X frequency and is very useful for examining protein complexes where parts of the 2D NOESY spectrum can suffer from problems of signal overlap.[98] Using these 2D and 3D approaches we have also examined complexes of *L. casei* DHFR with trimethoprim (**4**) labeled at the [1,3,2-amino-^{15}N] or [7,4′-OCH_3-^{13}C] positions,[17] and have defined details of the trimethoprim binding site from the measured NOEs. The ^{15}N-edited NOEs detected in the 2D HMQC-NOESY $^1H/^{15}N$ spectrum of the former complex are shown in Figure 6.[17]

$^{12}C/^{13}C$-filtered 2D NOESY experiments also provide a means for detecting NOEs from protons in an unlabeled ligand to protons attached to ^{13}C in a ^{13}C-labeled protein: Figure 7 shows the ligand – protein NOE connections involving the N10-CH_3 group and the α-CH protons of unlabeled methotrexate bound to ^{13}C-labeled *L. casei* DHFR.[19] All NOEs between protons attached to ^{13}C have been rejected. Similar experiments using $^{14}N/^{15}N$-filtered NOESY sequences have been used to detect NOEs between NH protons in unlabeled ligands and protons in ^{15}N-labeled DHFR.[99]

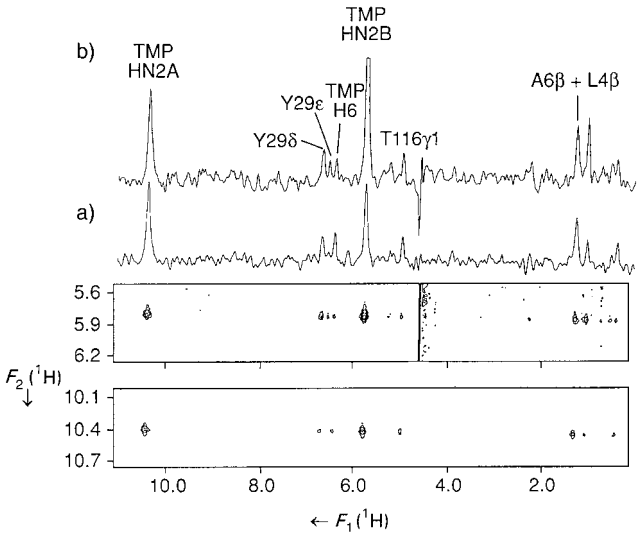


Figure 6. Part of the spectrum from an ^{15}N-edited 2D NOESY experiment on the complex of *L. casei* DHFR with [1,3,2-amino-^{15}N]trimethoprim (edited at a ^{15}N frequency of $\delta = 59.6$ from external NH_4Cl). The upper traces are the rows at the frequencies of the HN2A and HN2B protons showing both intramolecular NOEs and intermolecular NOEs. (Reprinted with permission from Martorell et al.[17])

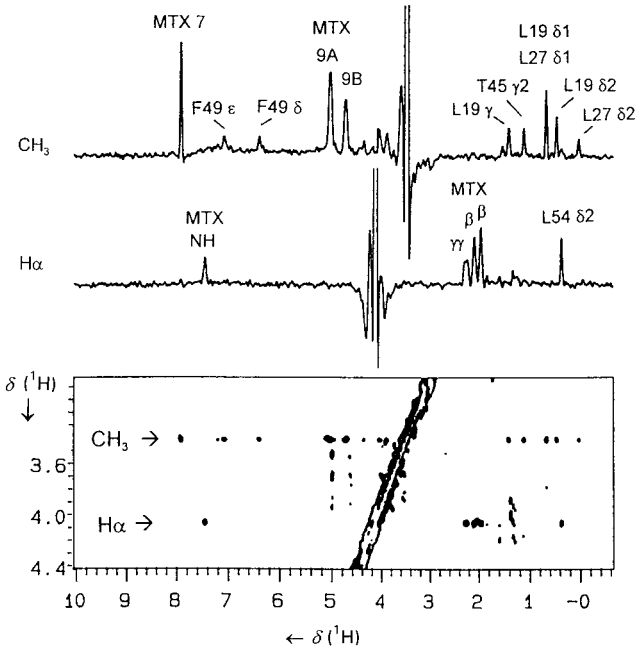


Figure 7. Part of the ^{12}C/^{13}C-filtered 2D NOESY spectrum acquired with a sample containing unlabeled methotrexate and ^{13}C-labeled *L. casei* dihydrofolate reductase (at 308 K and pH 6.5) showing the NOEs involving the methotrexate N10-CH_3 and Hα protons. (Reprinted with permission from Gargaro et al.[19])

Figure 8 shows the extensive set of ligand – protein NOEs determined for **5** in its complex with *L. casei* DHFR.

For nonlabeled ligands it is sometimes possible to take advantage of dynamic processes within the bound ligand to assist in making protein – ligand NOE assignments. Several NOEs were obtained with this novel approach by monitoring the effects of temperature on NOE signals that result from dynamic processes in trimetrexate (**6**) bound to DHFR.[20] At 278 K the trimethoxy ring of bound trimetrexate is flipping

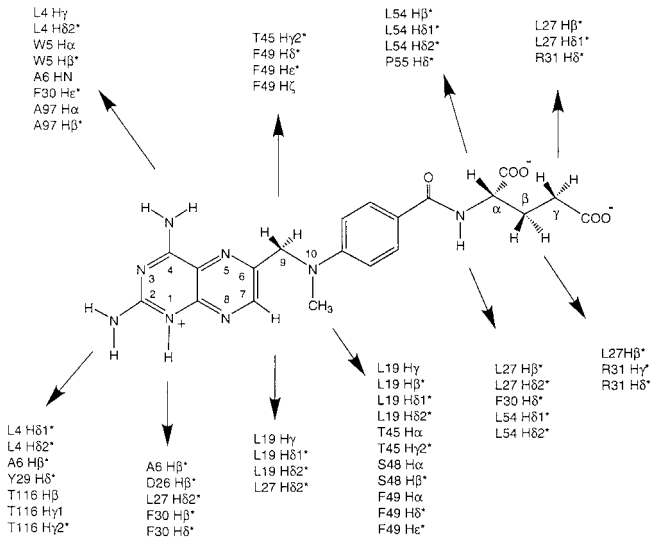


Figure 8. Intermolecular protein – ligand NOEs in the DHFR – methotrexate complex. (Reprinted with permission from Gargaro et al.[19])

sufficiently slowly to give separate narrow signals for the 3′- and 5′-OCH_3 groups in slow exchange and these give good NOE cross-peaks to protein signals. At higher temperatures the ligand signals broaden because of the increase in flip rate and their NOE cross-peaks disappear, thus allowing the corresponding signals that are observed only in the lower temperature spectrum to be identified as NOEs involving ligand protons.

4. Structure Determinations of DHFR Complexes

In recent years NMR has become a powerful method for determining the structures of proteins in solution.[59, 111] Once the signal assignments are known it is possible to extract the NOE and coupling constant information from the NMR spectra and to use these to obtain a list of proton – proton distance and angle constraints. Simulated annealing molecular dynamics calculations can then be used to obtain models of protein structures consistent with these constraints.[112, 113]

We have used these methods to determine the three-dimensional structures of complexes of inhibitors **4** – **7** with *L. casei* DHFR.[17–20] Stereospecific assignments and torsion angle constraints were deduced from NOE and coupling constant data using the program AngleSearch.[114] Several ^{13}C and ^{15}N analogues of trimethoprim (**4**) and methotrexate (**5**) were used to obtain the isotope-edited or isotope-filtered spectra required for defining the protein – ligand NOEs. The structures of the complexes were determined using either conventional simulated annealing molecular dynamics calculations or by using various docking methodologies. For example, the three-dimensional solution structure of the complex of *L. casei* DHFR with the anticancer drug methotrexate (**5**)[19] was determined using 2531 distance, 361 dihedral angle, and 48 hydrogen bond restraints obtained from analysis of multidimensional NMR spectra. Simulated annealing calculations produced a well-defined family of structures fully consistent with the constraints (Figure 9a).

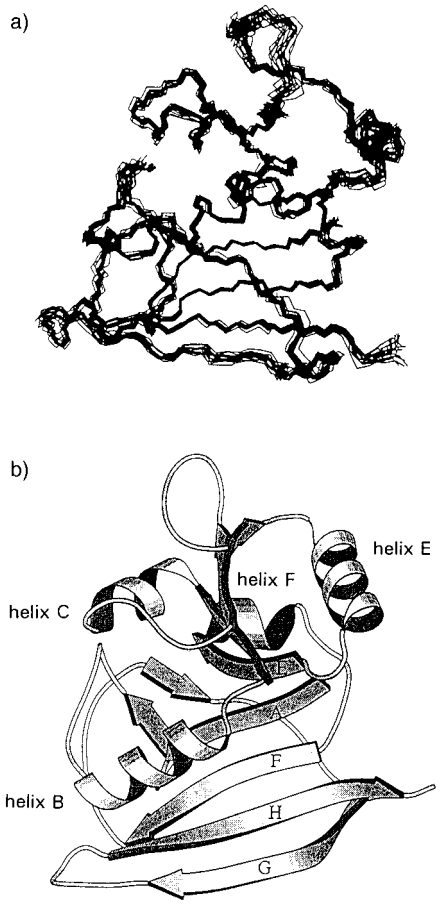


Figure 9. a) Superposition of the backbone atoms of residues 1–162 from the final 21 energy-minimized structures of the *L. casei* DHFR–methotrexate complexes.[19] b) MOLSCRIPT diagram[172] of the representative structure of this complex showing the elements of secondary structure. The representative structure is the one closest to the average structure. (Reprinted with permission from Gargaro et al.[19])

The structure has four α-helices as well as eight β-strands with two other regions, comprising residues 11–14 and 126–127, which also interact with each other in a β-sheet manner (Figure 9b). The overall fold of the binary complex in solution is very similar to that observed in the X-ray studies of the ternary complex of *L. casei* DHFR formed with methotrexate (**5**) and NADPH[14] (Figure 10).

To obtain high quality structures of the ligand binding site region it is necessary to have a large number of protein–ligand distance constraints (see Figure 8). In earlier simulation studies[115] using NMR-type distance constraints (classified as strong, medium, and weak) extracted from an X-ray structure of folate bound to human DHFR,[16] we found that molecules of this type require at least 50 ligand–protein distance constraints to produce acceptable structures. In the case of the binding site determined for methotrexate (**5**), the pteridine ring part is very well-defined (see Figure 10), but the structure around the glutamate moiety needed to be improved by including restraints reflecting the previously determined[116, 117] specific interactions between the glutamate α-carboxylate group with Arg57 and the γ-carboxylate group with His28 (see Section 5). The NADPH binding site appears to be essentially preformed in the binary complex and this

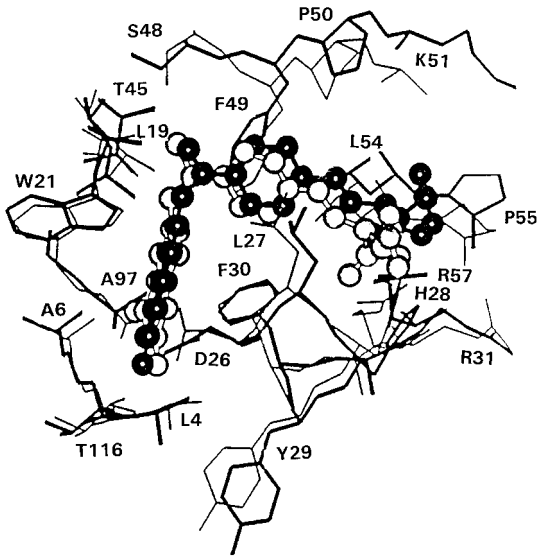


Figure 10. A comparison of the methotrexate binding site in the NMR-determined solution structure (dark lines) in the *L. casei* DHFR–methotrexate structure with that in the crystal structure (light lines) of the ternary complex DHFR-methotrexate-NADPH (crystal data from Bolin et al).[14] (Reprinted with permission from Gargaro et al.[19])

may contribute to the tighter binding of the coenzyme in the presence of methotrexate since no major conformational change is required when NADPH binds to the binary complex.

Structures of other *L. casei* DHFR complexes, such as that with trimetrexate (**6**), have also been determined and one can begin to make comparisons of the similarities and differences between the modes of binding of different ligands.[20] Figure 11 shows a superposition of methotrexate (**5**) and

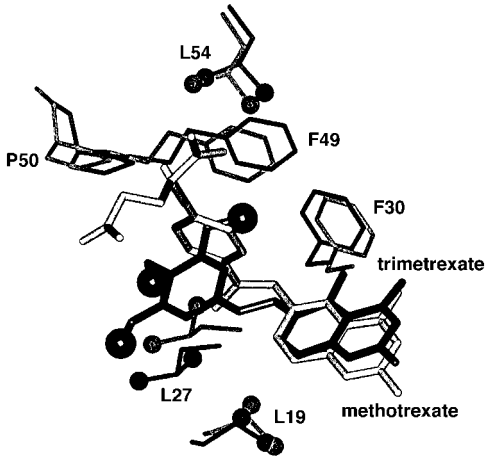


Figure 11. A comparison of the trimetrexate (dark shading) and methotrexate (light shading) binding sites in the structures of their *L. casei* dihydrofolate reductase complexes. The superposition of structures was made over the backbone atoms (N, Cα, and C) of all 162 residues in the two structures. Methyl groups are shown as balls. (Reprinted with permission from Polshakov et al.[20])

trimetrexate (**6**) in their binding sites in DHFR. It can be seen that the 2,4-diaminopyrimidine moieties of the two drugs bind in the same binding pocket. The remaining parts of the

molecules adapt their conformations such that they can make effective van der Waals interactions with essentially the same set of hydrophobic amino acids, whose side-chain orientations and local conformations are not greatly changed in the two complexes.

In addition to the conventional structural determinations carried out using simulated annealing calculations we have been exploring various methods for docking ligands into a reference protein structure.[17, 18] The atomic coordinates used for a reference protein are usually obtained from an X-ray structure of a related complex containing the same protein. Recently we have been using NMR-determined structures of a related complex in the docking procedure: in this approach NMR constraints from the reference structure are used rather than the atomic coordinates of the protein.[118] The method uses a set of experimentally determined values for protein – ligand, ligand – ligand, and protein – protein restraints for residues in or near to the binding site combined with a set of protein – protein restraints involving all the other residues which are taken from the list of restraints previously used to generate the reference structure of a related complex. The binding site residues that are influenced by replacing the reference ligand by the new ligand are determined by monitoring differences in ^{1}H chemical shifts between the new complex and the reference complex. The method has been validated by showing the excellent agreement between structures of the *L. casei* DHFR – trimetrexate complex calculated by the conventional method using a full experimentally determined set of restraints and that using this new restraint-docking method based on a *L. casei* DHFR – methotrexate reference structure.[118]

5. Detection of Specific Interactions

Protein – ligand complexes often contain specific interactions between charged groups on the interacting molecules, and NMR spectroscopy has proved to be an excellent method for determining the ionization states of such groups and for monitoring how they are perturbed on forming the complex. Changing the ionization state of a group can result in large changes in the chemical shifts of nearby nuclei and these are particularly easy to characterize for nuclei such as ^{13}C, ^{15}N, and ^{31}P. For example, ionization states of phosphate groups can be conveniently measured from ^{31}P chemical shift measurements. The 2′-phosphate groups of $NADP^{+}$ and NADPH in the coenzyme complexes of DHFR have been shown to bind in their dianionic forms (for both *L. casei* and *E. coli* DHFR) with the pK of the 2′-phosphate in the bound coenzyme group being decreased by at least 3 units compared to its pK value in the free species.[108] The difference $\Delta\delta$ in ^{31}P chemical shift between the mono- and dianionic species is about 3.5, which makes it easy to identify the bound form as being in the dianionic state.

More usually, one examines samples that have been specifically labeled with isotopes for such studies. For example, Blakley and co-workers[49] have used ^{15}N chemical shift measurements to show that the N3 atom of [2-amino,3-^{15}N]folate bound to human DHFR exists as the protonated unionized form even at pH 9.5. In earlier experiments on complexes of [2-^{13}C]folate with *Streptococcus faecium* DHFR, the observed ^{13}C chemical shifts indicated that the bound folate is in the same protonation state as free folate.[119]

5.1. Protein Interactions Involving the Pyrimidine Ring of the Inhibitor

A consideration of the crystal structure of the DHFR-methotrexate complex[14] indicated that a protonated N1 position of the pteridine ring would be sufficiently close to the carboxylate group of the conserved Asp 27 to form a hydrogen bond. Cocco and co-workers[119] have used NMR spectroscopy to characterize this interaction. They examined the ^{13}C NMR spectrum of [2-^{13}C]methotrexate bound to *S. faecium* DHFR and from measurements of the characteristic ^{13}C shift of the C2 carbon atom they showed that the adjacent N1 is protonated in the complex and remains protonated even at pH 10. Thus, by combining results from NMR (to give the protonation state of N1) and X-ray studies (to indicate the interacting residue, Asp 27), a more complete picture of this particular interaction could be obtained. Similar experiments have been carried out with *L. casei*[46] and *E. coli* DHFRs and two *E. coli* mutant enzymes in which Asp 27 was replaced by asparagine and serine.[120] While the pK value of methotrexate is greater than 10 in the complex with the wild-type enzyme, the pK value is less than 4 in complexes with the mutant enzymes. Cannon and co-workers[121] have predicted the pK_a value for the N1 position in the DHFR – methotrexate complex and their quantum chemistry calculations suggest that N1 remains unprotonated and forms a hydrogen bond with an unionized carboxylate group on the conserved Asp residue, which is in contradiction to the experimental results. While the experimental evidence for N1 protonation appears to be strong, the definitive experiment of examining the DHFR complex with [1-^{15}N]labeled methotrexate has not yet been carried out: a protonated N1 would be detected as a ^{15}N and/or ^{1}H signal with a 90 Hz doublet splitting from ^{1}H-^{15}N spin coupling.

Measurements of ^{13}C chemical shifts of [2-^{13}C]trimethoprim complexed with *L. casei* DHFR have revealed that the pyrimidine N1 is protonated in bound trimethoprim.[92, 122] Figure 12 shows that the ^{13}C chemical shift of the bound species is similar to that of the free protonated N1 species and the pK of the bound trimethoprim is decreased by at least 3 pK units.

The protonated state of N1 was demonstrated even more directly in ^{1}H NMR experiments on a complex of [1,3,2-amino-^{15}N]trimethoprim with the enzyme where it was possible to detect the ^{1}H signal of the directly bonded N1 proton (the signal was observed at $\delta = 14.4$ and had a 90 Hz doublet splitting characteristic of ^{1}H-^{15}N spin coupling).[92] The ^{15}N spectra also clearly showed that N1 is protonated (the ^{15}N chemical shift of N1 changes by $\delta \approx 80$ on protonation).

Hydrogen-bonding interactions involving the 2-NH_2 and 4-NH_2 protons of trimethoprim (**4**) have also been detected by

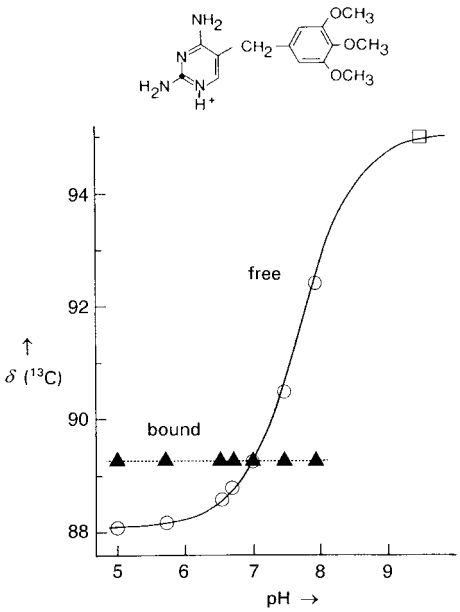


Figure 12. The NMR titration curve (^{13}C chemical shifts versus pH) of [2-^{13}C]trimethoprim: (○) free ligand; (▲) in its complex with *L. casei* DHFR. The solid line is calculated for a p$K = 7.70$ and $\Delta\delta = 7.09$. The data point at pH 9.5 (□) was obtained for free trimethoprim in the absence of enzyme. (Reprinted with permission from Roberts et al.[122])

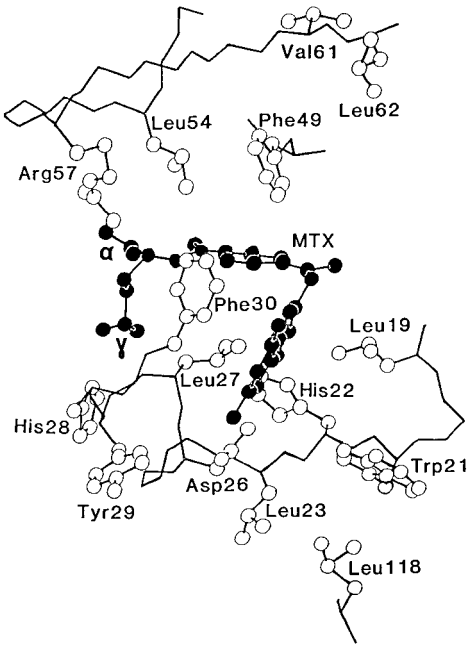


Figure 13. Part of the crystal structure of the *L. casei* DHFR-MTX-NADPH complex (from the data of Bolin et al.[14]) showing residues around the methotrexate binding site. Heavy atoms of methotrexate are shown as black balls and those of selected side chains as white balls. (Reprinted with permission from Hammond et al.[159])

measuring ^{1}H/^{15}N chemical shifts in DHFR complexes with ^{15}N-labeled trimethoprim. Dale and co-workers[123] used this approach in their studies of a trimethoprim-resistant mutant DHFR (Phe 98 →Tyr) from *S. aureus* to show that the 4-NH_2 group of trimethoprim in the complex with the mutant enzyme and NADPH no longer forms a hydrogen bond with the carbonyl oxygen atom of Leu 5.

5.2. Protein Interactions Involving the Glutamic Acid Moiety of Folate Analogues

In the crystal structure studies by Matthews and co-workers[13, 14] on the ternary complex of *L. casei* DHFR with methotrexate (**5**) and NADPH the α-carboxylate group of the glutamic acid moiety of methotrexate is found to be positioned such that it could form an ion pair with the guanidino group of the conserved Arg 57 while the γ-carboxylate group was located such that it could interact with the imidazole ring of His 28 as shown in Figure 13. We have shown that NMR methods can be used to detect the presence of these interactions in complexes formed with ligands containing carboxylate groups.

5.2.1. Interactions of Arg 57 with Ligand Carboxylate Groups

Recent studies of ^{1}H/^{15}N signals from arginine residues have proved to be particularly useful for monitoring protein–ligand interactions.[124–126a] For example, in the ^{1}H/^{15}N HSQC spectra of the complex of *L. casei* DHFR with methotrexate, the interaction of the methotrexate α-carboxylate group with the guanidino group of Arg 57 can be directly detected by using such measurements. In this case, the interaction causes large shift perturbations for two of the guanidino NH$^{\eta}$ protons and hindered rotation about the N$^{\varepsilon}$-C$^{\zeta}$ and C$^{\zeta}$-N$^{\eta}$ bonds of the guanidino group (Figure 14 a) then results in four separate

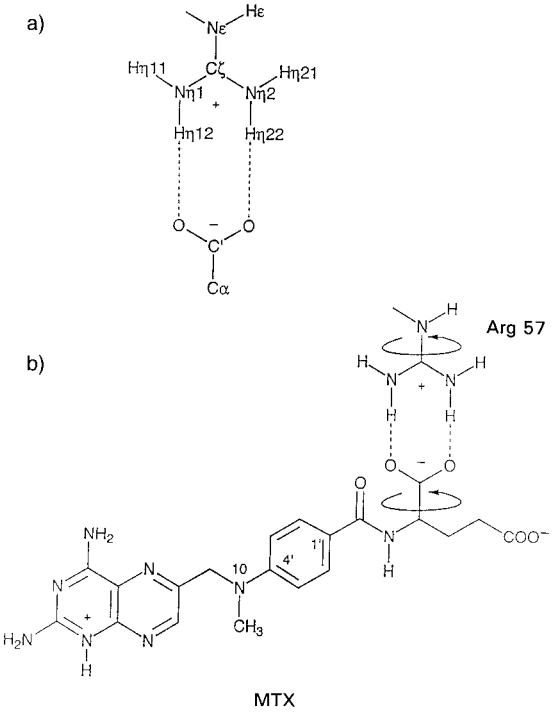


Figure 14. a) Symmetrical end-on interaction of the α-carboxylate oxygen atoms of methotrexate with the guanidino group of Arg 57. b) Correlated rotations about the N$^{\varepsilon}$-C$^{\zeta}$ bond of the Arg 57 guanidino group and the C′-C$^{\alpha}$ bond of the glutamate α-carboxylate group of methotrexate (**5**, MTX) in its complex with *L. casei* DHFR. (Reprinted with permission from Nieto et al.[125])

signals for the NH^{η} nuclei being observed in the HSQC spectra (Figure 15). All four signals could be specifically assigned to their particular NH^{η} protons in Arg57. The large downfield chemical shifts observed for two of these signals indicated that two of the NH^{η} protons ($NH^{\eta 12}$ and $NH^{\eta 22}$) form hydrogen bonds with the charged oxygen atoms of the α-carboxylate group of the glutamate moiety of methotrexate.

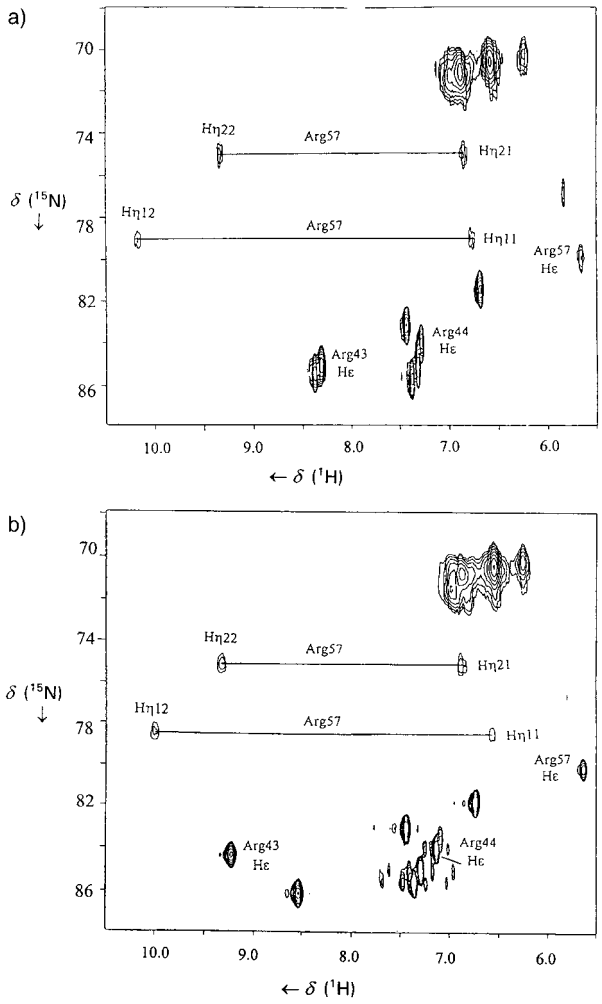


Figure 15. The ^{1}H-^{15}N HSQC spectrum of the Arg57 NH^{η} protons (274 K) from a) the DHFR – methotrexate complex; b) the DHFR-methotrexate-NADPH complex. The chemical shifts indicate a symmetrical end-on interaction of the α-carboxylate oxygen atoms of methotrexate with the guanidino group of Arg57 as indicated in the structure shown in Figure 14a. (Reprinted with permission from Gargaro et al.[124] and Nieto et al.[125])

Because only the two centrally situated NH^{η} protons are involved in the hydrogen bonding, the guanidino group must be forming part of a symmetrical end-on interaction as shown in Figure 14a.[124] These effects involving Arg57 were not seen for complexes with ligands such as trimethoprim (**4**) that do not contain any carboxylate groups.

The $^{1}H/^{15}N$ HSQC spectrum given in Figure 15 showing the four resolved NH^{η} signals of Arg57 for the DHFR – methotrexate complex was recorded at 274 K: increasing the temperature caused line-broadening and coalescence of signals as a result of exchange effects.[125] The rotation rates for the N^{ε}-C^{ζ} and C^{ζ}-N^{η} bonds were calculated from lineshape analysis and, for the N^{ε}-C^{ζ} bond, from 2D *zz*-HSQC exchange experiments[126b,c] carried out using a pulse sequence described by Yamazaki and co-workers.[126a] These experiments allow the observation of the transference of heteronuclear *zz*-magnetization between the different ^{15}N-labeled sites. Interactions between the methotrexate α-carboxylate group and the Arg57 guanidino group decrease the rotation rates for the N^{ε}-C^{ζ} bond by about a factor of 3 and those for the C^{ζ}-N^{η} bonds by a factor of more than 300 compared with their values in free arginine.[126a–128]

Surprisingly, the relative rates of rotation about these two bonds are reversed in the protein complexes relative to their values in free arginine (Figure 16). This behavior can readily

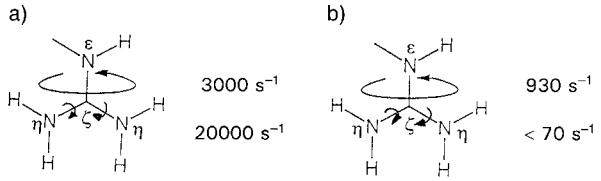


Figure 16. Rotation rates at 313 K for the N^{ε}-C^{ζ} and C^{ζ}-N^{η} bonds in a) free arginine residues; b) Arg57 in the DHFR – methotrexate complex.

be explained if there are correlated rotations about the N^{ε}-C^{ζ} bond of the Arg57 guanidino group and the C′-C^{α} bond of the glutamate α-carboxylate group of methotrexate (see Figure 14b). These correlated rotations would allow rotation about the N^{ε}-C^{ζ} bond without the need to break the interactions of the NH^{η} protons with the carboxylate oxygen atoms. The latter interactions would then slow down rotations about the C^{ζ}-N^{η} bonds preferentially.[125]

In the $^{1}H/^{15}N$ HSQC spectrum of the DHFR ternary complex formed with methotrexate and NADPH (Figure 15b) the NH^{ε} proton of Arg43 shows a large downfield chemical shift and also a retardation of its rate of proton exchange with water compared with the binary complex with methotrexate.[124] Resolved signals for the four NH^{η} protons of Arg43 were not observed. This behavior contrasts with that seen for Arg57 and is consistent with the presence of a side-on interaction of the guanidinium NH^{ε} proton with the charged oxygen atoms of the ribose 2′-phosphate group of NADPH. It was seen earlier that ^{31}P NMR studies indicate that the 2′-phosphate group binds to DHFR in its dianionic form both in solution[108] and in the solid state.[129]

5.2.2. Interactions of His28 with Carboxylate Groups

In our previous NMR studies of the ^{1}H NMR chemical shifts of the imidazole C2 proton of histidine in complexes of the *L. casei* DHFR with methotrexate (**5** and several other ligands containing the glutamic acid moiety) we found that the p*K* value of His28 was ca. 1 p*K* unit higher than its value in the ligand-free enzyme (Figure 17)[130, 131] This value reflects the interaction of His28 with the MTX γ-carboxylate group (Figure 13) and provides an excellent method for monitoring interactions involving His28.[132] We have used these effects to study complexes of *L. casei* DHFR with the α- and γ-amide

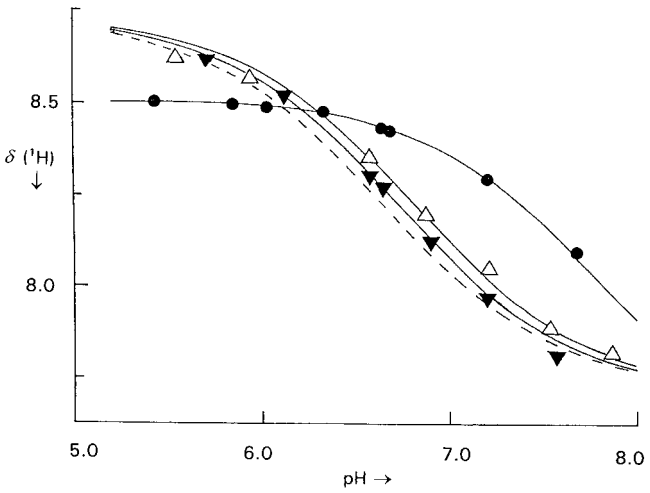
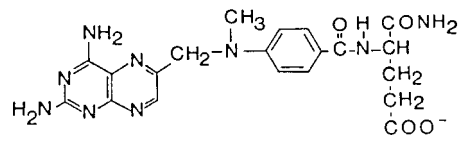


Figure 17. The pH dependence of the chemical shift (^{1}H, referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate) of the C2 protons of His 28 in several dihydrofolate reductase complexes. (●) methotrexate (**5**); (▼) methotrexate γ-amide (**10**); (△) methotrexate α-amide (**9**); (–––) free enzyme.[116] (Reprinted with permission from Feeney.[23])

analogues of methotrexate, **8** and **9**.[116] The γ-amide and α-amide analogues bind one and two orders of magnitude less tightly to *L. casei* DHFR, respectively, than does methotrexate. For the γ-amide MTX complex with DHFR, the ^{1}H chemical shifts of the His 28 imidazole C2 proton indicated



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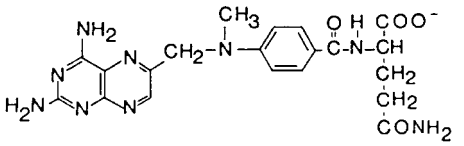

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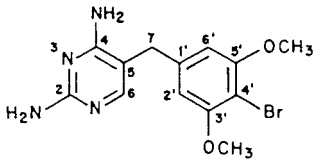
that its pK value is not perturbed from its value in the ligand-free enzyme showing that an ion-pair interaction is not formed (see Figure 17).[116]

The ^{1}H NMR spectrum of the α-amide MTX complex with DHFR also showed that the pK of His 28 was not perturbed even though the γ-carboxylate group is available for interaction. Thus, modification of the α-carboxylate not only destroys the Arg 57 interactions, but also perturbs the overall structure such that the available free γ-carboxylate can no longer form an ion pair with His 28. Changes in the shielding of several protons near to the benzoyl ring also accompany the removal of the α-CO_2^- interaction with Arg 57. These differences in chemical shift are consistent with a change in the orientation of the benzoyl ring, which results in changes in the ring-current shielding effects at neighboring protons.[116, 133]

6. Rational Design of Inhibitors

The detailed structural information available for complexes of dihydrofolate reductase[13–20] has been used in combination with molecular graphics and molecular modeling methods to design improved inhibitors.[12, 45, 133–144] NMR spectroscopy is proving to be an excellent alternative method to X-ray crystallography[140] for monitoring the design process by detecting specific interactions between a new inhibitor and the protein, and assessing whether or not the predicted interactions have taken place.[25, 116, 117, 143]

We have designed improved DHFR inhibitors by preparing analogues of trimethoprim (**4** or brodimoprim **10**, in which a bromo substituent replaces the 4′-OCH_3 group) that have side chains on the O3′ position of the benzyl ring to make additional interactions with certain protein residues that are normally involved in substrate binding but not used for binding to **4**. For example, when the 2,4-diaminopyrimidine ring of a brodimoprim analogue is modeled into its binding site in DHFR it is seen that the brodimoprim, unlike folate or methotrexate, cannot make any direct interactions with Arg 57 and His 28 (Figure 18).[117]



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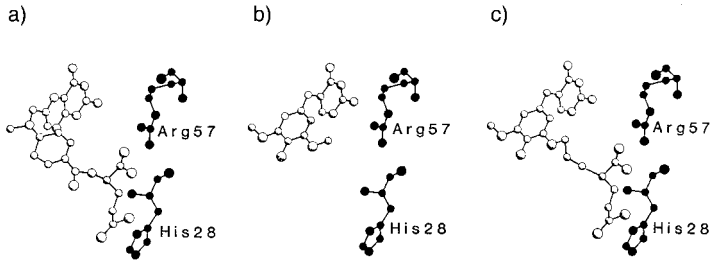
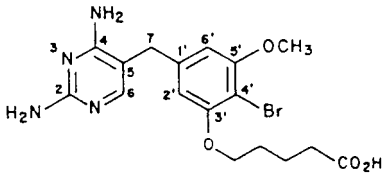


Figure 18. a) Detail from the X-ray crystal structure of the complexes of *L. casei* DHFR with methotrexate (**5**) and NADPH (Bolin et al.[14]). The proximity of the α- and γ-carboxyl groups of methotrexate to Arg 57 and His 28 are clearly visible. b) Brodimoprim (**10**) in its bound conformation makes no contacts with these residues. c) A model of the mode of binding of the dicarboxylate derivative **7** of brodimoprim (**10**)[117] using the crystal structure data of Matthews and co-workers.[13, 14] (Reprinted with permission from Feeney.[25])

In collaboration with colleagues from Hoffmann-LaRoche we have synthesized several analogues of brodimoprim (**8**) that were designed to interact with these additional residues.[117] These rationally designed analogues have an additional side chain containing one or two carboxylate groups aimed at interacting with the Arg 57 and/or His 28 residues on the protein. The measured inhibition constants for the various analogues (Table 1) provide estimates of their improved binding. The ^{1}H chemical shifts of the His 28 imidazole C2 protons were used to monitor the pK values of His 28 residues, and ^{1}H/^{15}N chemical shifts of the Arg 57 guanidino groups were used to monitor interactions with ligand carboxylate groups in the various DHFR complexes.[143] These experi-

Table 1. Inhibition constants (K_i) and pK values of His 28 for complexes of *L. casei* DHFR with brodimoprim and some of its analogues.

Ligand	K_i [nM]	pK His 28
10	11.3	6.80
11	0.2	6.83
12	0.6	6.80
7	< 0.01	7.80



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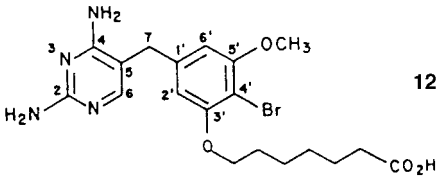

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ments allowed us to assess if the ligand side chains are binding in the predicted manner. One example, the carboxybutyl derivative **11**, was designed to interact with the Arg 57 residue. The observed perturbations to the $^1H/^{15}N$ chemical shifts of the Arg 57 guanidino group nuclei are those expected for a symmetrical end-on interaction of the carboxylate group with the Arg 57 guanidino protons. This interaction results in four separate NH^{η} signals in the HSQC spectrum, two of which (one from each NH_2 group) have substantial downfield shifts as a result of hydrogen bonding.[143] As expected for this analogue, there was no perturbation of the pK value of His 28.

Another ligand, the dicarboxy derivative **7**, was designed to interact with both His 28 and Arg 57. Its complex with DHFR showed an increase in the pK value of His 28 of about 1 unit (see Table 1), which indicated that a carboxylate-His 28 interaction had been made (see Figure 18). The interaction of a carboxylate group with the Arg 57 guanidino group was also confirmed by the appearance of the four NH^{η} signals in the $^1H/^{15}N$ HSQC spectrum, which indicate a symmetrical end-on interaction with the carboxylate oxygen atoms and the presence of hindered rotation within the guanidino group (see Figure 15). The dicarboxyl derivative **7** binds three orders of magnitude more tightly to DHFR than does the parent molecule and retains its specificity of binding with respect to human DHFR.

Some of the other brodimoprim analogues do not bind in the expected manner. For example, the carboxyhexyl derivative **12** (see Table 1), which was designed to achieve the carboxylate – His 28 interaction, did not perturb the pK value of His 28 even though it binds fairly tightly to the enzyme. Thus, although **12** can reach His 28, it appears to prefer to bind at some alternative site. The monitoring of His 28 and Arg 57 NMR signals clearly provides a convenient and direct method for assessing whether or not a predicted interaction has taken place in these complexes.[117, 143]

7. Dynamic Processes in Protein – Ligand Complexes

Analysis of ^{15}N and ^{13}C relaxation data can provide information about rapid segmental motions in the enzyme – ligand complexes (rates of 10^9 to 10^{12} s^{-1}), while line-shape analysis and transfer of magnetization experiments can characterize intramolecular motions at much slower rates (rates of 10^0 to 10^4 s^{-1}), typical of those observed for bond rotations in arginine residues, for ring-flipping of aromatic rings, and for breaking and reforming of hydrogen bonds.

7.1. Rapid Motions

Epstein and co-workers[145] have used ^{15}N relaxation measurements on the complex of *E. coli* DHFR with folate (**1**) to determine the dynamics of the backbone and tryptophan side chain. They found large amplitude motions for the backbone on the pico- and nanosecond time scales in regions of the protein that could be implicated in transition-state stabilization. They concluded that these time-dependent structure variations could be associated with the catalytic properties of the enzyme.[145] Relaxation time studies on complexes of the *L. casei* enzyme have also been reported.[20, 93] By analyzing the ^{13}C relaxation times of the C7 carbon atom in [7,4′-OCH_3-$^{13}C_2$]trimethoprim—bound to *L. casei* DHFR—the overall correlation time (tumbling time) of the molecule was calculated to be 15.4 ns at 295 K. The 4′-OCH_3 carbon atom has a sixfold slower relaxation rate. This can be interpreted in terms of additional rapid motions that affect the relaxation of the 4′-OCH_3 carbon atom and suggests that the benzyl ring is librating rapidly ($> 10^{10}$ s^{-1}) over angles of $\pm 30^{\circ}$.[93]

7.2. Slow Intramolecular Processes

Examples of bond rotations and correlated rotations have already been discussed for the case of arginine guanidino groups interacting with carboxylate groups in ligands. Correlated rotations have also been seen for the ring-flipping of *para*-substituted aromatic rings in bound ligands. In complexes of *L. casei* DHFR with ligands containing symmetrically substituted aromatic rings, the rings are usually undergoing ring-flipping. NMR spectroscopy is the ideal technique for determining the rates of such processes. In some complexes, such as that with folate (**1**), the rate of ring-flipping is fast enough to ensure that only a single signal is seen for the 2′,6′-protons (and another for the 3′,5′-protons) of the benzoyl ring. For other complexes, such as the DHFR – methotrexate complex, the ^1H signals for the corresponding protons have never been detected—probably because of line broadening as a result of exchange processes at the temperatures studied. However, by examining ^{19}F spectra of the complex of 3′,5′-difluoromethotrexate with DHFR at different temperatures it was possible to characterize the ring-flipping in detail (see Figure 19).[107]

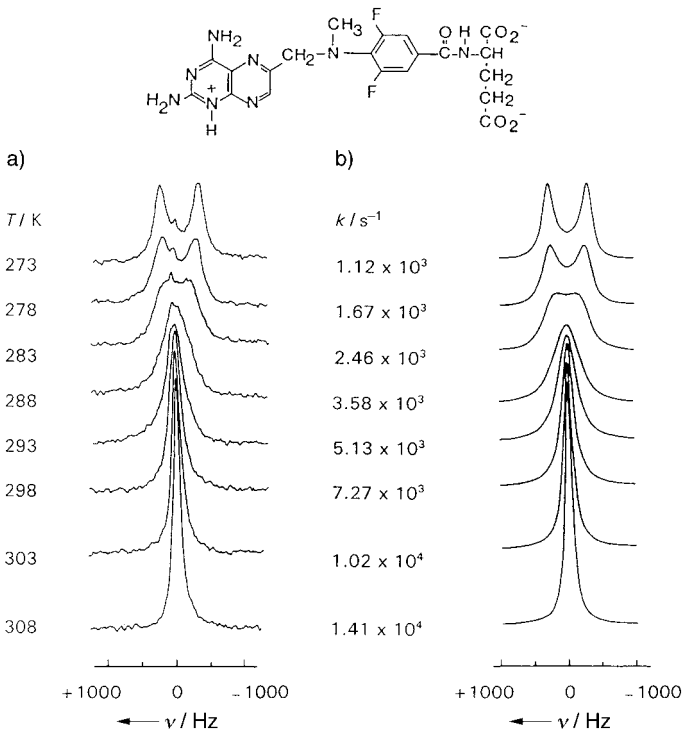


Figure 19. a) The 188.2 MHz ^{19}F NMR spectra of 3′,5′-difluoromethotrexate complexed with *L. casei* DHFR examined at a series of temperatures. b) The corresponding calculated spectra and rates of exchange. (Reprinted with permission from Clore et al.[107])

Two separate signals are seen for the 3′,5′-fluorine nuclei at 274 K, and on increasing the temperature the signals broaden and eventually coalesce (at ~298 K). A line-shape analysis of this classical 2-site exchange behavior gave the rate of ring-flipping as 7×10^3 s^{-1} at 298 K for the binary complex and 2×10^4 s^{-1} at 298 K for the ternary complex with NADPH. In both complexes the ring-flipping motions require correlated rotations about the bonds attached to the 1′- and 4′-positions of the ring. Verma and co-workers[147] have calculated the possible reaction pathway for the ring-flipping process in this complex.

Rates of ligand ring-flipping have also been determined in complexes of *L. casei* DHFR with trimethoprim (**4**),[93, 94] pyrimethamine analogues,[102] and with trimetrexate (**6**).[20] Line-shape measurements on ^{13}C spectra (and more recently from ^{1}H/^{13}C HMQC spectra[148]) of [3′-OCH$_3$-^{13}C-]trimethoprim have been used to characterize these rates for the benzyl ring in DHFR complexes with trimethoprim analogues.[93, 94] For the binary DHFR – TMP complex the rate of ring-flipping is 793 s^{-1} at 298 K. From a consideration of the bound conformation of trimethoprim it could be seen that rotation about the C7-C1′ bond (τ_2 in Figure 20) cannot occur without changing the C5-C7 torsion angle (to remove steric interactions between the pyrimidine and benzyl rings). This in turn requires a fluctuation in the protein structure such that the torsion angle about the C5-C7 bond (τ_1 in Figure 20) in bound trimethoprim needs to change by at least 35° in order to remove this steric hindrance and allow the ring-flipping to take place about τ_2.[93]

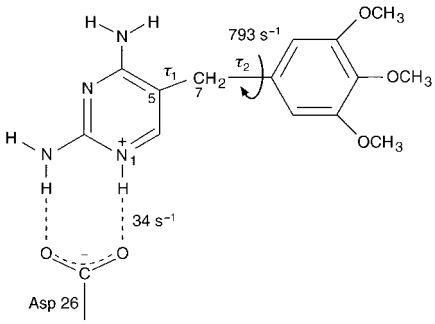


Figure 20. Dynamic processes in the complex of trimethoprim with *L. casei* DHFR measured at 298 K. The lifetime of the complex is 0.5 s at this temperature. The flipping rate was measured from line-shape analyses over a range of temperatures[148] and corrects the value given in ref. [93].

Analyses of the line widths of NH proton signals measured as a function of temperature provide rates of proton exchange with the solvent. These can be related to the rates of breaking and reforming of hydrogen bonds in cases where hydrogen-bond breaking is a required step before proton exchange with the solvent can take place.[92] The rate of breaking the hydrogen bond between the trimethoprim pyrimidine N1 proton and the carboxylate oxygen of Asp26 in the DHFR – trimethoprim complex was estimated as 34 s^{-1} at 298 K. At this temperature the dissociation rate for the complex (2 s^{-1}) is much slower than either the frequency of ring-flipping (793 s^{-1}) or the hydrogen-bond breaking (34 s^{-1}, see Figure 20). It is clear that both processes involve the breaking and reforming of protein – ligand interactions many times within the lifetime of the complex. The lifetimes of the partially dissociated species will be very short and complete dissociation would require the events to occur simultaneously or in close succession.

This picture would be consistent with our earlier suggestion concerning a "zipper"-type mechanism for binding and releasing flexible ligands in protein – ligand complexes.[149] In this model the binding of a flexible ligand to a protein was proposed to occur by the initial formation of a nucleation complex where the ligand is only partially bound and which then undergoes a series of conformational rearrangements that lead to other interactions with the protein forming in a zipper-type process to give the fully bound complex (Figure 21). Dissociation would involve a similar stepwise reversal of the various partially bound states. Such processes would allow for rapid rates of association and dissociation (by partitioning the overall activation energies into a series of smaller values) while retaining the specificity of binding that results from the multi-site interactions between the flexible ligand and the protein.

8. Multiple Conformations

NMR spectroscopy has proved to be a powerful technique for detecting multiple conformations in protein – ligand complexes in solution. In many cases two or more conformations have been detected by the observation of separate NMR spectra for the different forms. The easiest way to detect

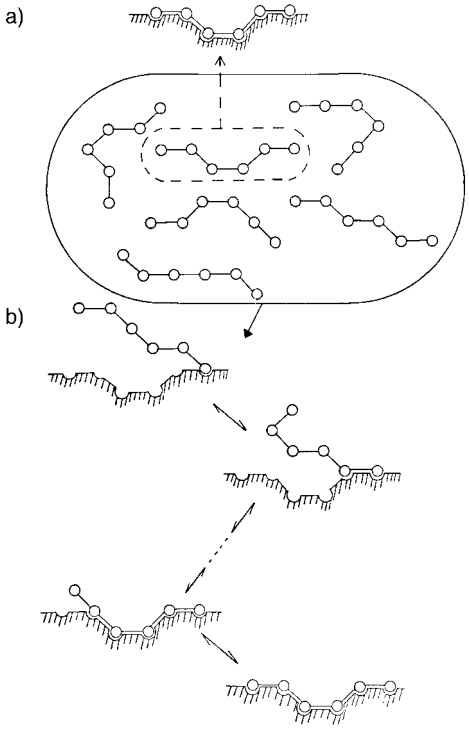


Figure 21. Schematic representation of a) the "lock and key" and b) the "zipper" model for ligand binding. Only those ligands which instantaneously have the correct conformation can bind in the "lock and key" model but essentially all the ligands can bind as in (b), where the final bound conformation is adopted by a step-wise "zipper-type" process. (Reprinted with permission from Burgen et al.[149])

multiple conformations is by examining NMR spectra from complexes formed with isotopically labeled ligands (^{3}H, ^{13}C, ^{15}N, ^{19}F), which readily reveal the multiplicity of signals when different conformations are present.

Multiple conformations found in protein – ligand complexes are often caused by the ligand being bound in more than one way. However, they have also been observed in the free enzymes. For example, NMR studies on DHFR from *E. coli* have revealed two distinct conformations which differ in the region of a mobile loop centered on residues 16 – 20.[150] Several complexes formed with dihydrofolate reductase from *L. casei*, *E. coli*, and *S. faecium* have been shown by NMR studies to exist as mixtures of conformations.[79, 90, 94, 103, 151–160] In such cases the relative amounts of the conformers in the equilibrium can be estimated from measurements of the intensity of the NMR signals from the different conformations. The conformational equilibria can sometimes be perturbed by changes in temperature, pH, or structure (for example, changes in the amino acid sequence by site-directed mutagenesis). The rate of interconversion between the conformational states of some complexes can be measured from analysis of the line shapes or, in favorable cases, by transfer of magnetization methods.[161]

The occurrence of multiple conformations often depends on the source of the enzyme. For example, the complex of the *E. coli* enzyme with methotrexate exists as two conformations[91, 95, 160] whereas the same complex formed with the *L. casei* enzyme is a well-defined single conformation.[159] However, for other ligands, such as folate (**1**), it is the complex with the *L. casei* enzyme[46, 90, 156, 157] rather than the *E. coli* enzyme[150] that shows multiple conformations. Multiple conformations have also been reported in X-ray studies, but in these cases it is difficult to assess the affect of crystal-packing forces on the stability of the conformations.[137]

8.1. Ternary Complexes of *L. casei* DHFR with Trimethoprim and NADP$^+$

The ternary complex formed by *L. casei* DHFR with trimethoprim and NADP$^+$ has been shown to exist as a mixture of two almost equally populated conformations (Forms I and II).[156, 157] These conformations were first detected in the ^{1}H spectrum of the complex where six of the seven imidazole C2 protons from the histidines gave rise to two separate signals corresponding to the two forms (Figure 22).[156, 157] The pairs of histidine signals each coalesced

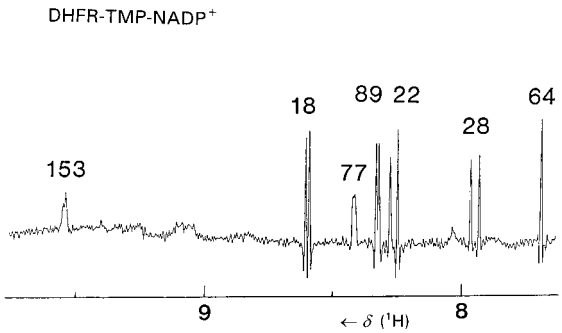


Figure 22. Part of the low-field region of the resolution-enhanced 500 MHz ^{1}H spectrum of the *L. casei* DHFR-Trimethoprim-NADP$^+$ complex showing the seven assigned C2-proton signals of histidine, six of which appear as doubled signals because of the presence of two conformations with almost equal populations. (Reprinted with permission from Birdsall et al.[157])

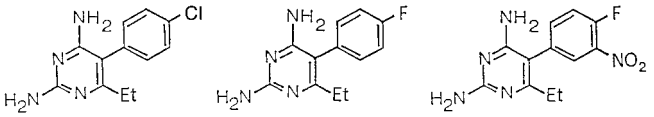
into single lines in a manner typical of a two-site exchange process on raising the temperature and a line-shape analysis at 314 K gave the rate of interconversion (18 s^{-1}) between the two forms.[156] Detailed ^{13}C and ^{31}P studies have confirmed the presence of the two conformations.[157] Two similar conformations are also seen in the DHFR-trimethoprim-NADP$^+$ complex formed with the *E. coli* enzyme.[96]

A consideration of the measured chemical shifts, coupling constants, and NOEs indicates that although the conformation of the bound trimethoprim is similar in the two forms that of the bound NADP$^+$ is very different: in Form II, the NADP$^+$ nicotinamide ring extends out into solution while in Form I it is buried within the protein.[156, 157] Conformational differences between Forms I and II were also detected in the nicotinamide ribose ring and the pyrophosphate linkages but the adenine ring and its ribose moiety are bound similarly in the two forms.

Complexes of various analogues of TMP and NADP$^+$ with DHFR were also shown to exist in two conformations with the populations of the two forms depending on the particular structures of the analogues.[157] Such information provides additional insights in studies of structure – activity relationships.

8.2. Complexes of *L. casei* DHFR with Pyrimethamine Analogues

In some complexes the multiple conformations result from flexible ligands binding in essentially the same binding site but in different conformational states. Examples are found in complexes formed with pyrimethamine analogues (such as **15**) containing asymmetrically substituted aromatic rings where two different rotational isomers of the bound ligand result



13 **14** **15**

from hindered rotation about the pyrimidine–phenyl bond.[102] We have used NMR spectroscopy to investigate such restricted rotation in complexes of *L. casei* DHFR with the pyrimethamine analogues **13**–**15**.[102] These analogues are of therapeutic interest both as antimalarial[162] and potential anti-tumour agents.[163, 164] The 3′-nitro-4′-fluoropyrimethamine analogue **15** has an asymmetrically substituted aromatic ring and can exist as a mixture of two rotational isomers (an enantiomeric pair; Figure 23).

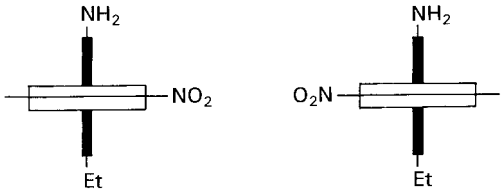


Figure 23. Schematic representation of the two rotational isomers (enantiomers) for 3′-nitro-4′-fluoropyrimethamine (**15**). The planes of the two aromatic rings are shown perpendicular to the plane of the paper and are presented as black and white bars. (Reprinted with permission from Birdsall et al.[102])

The ^{19}F NMR spectrum of the complex of **15** with DHFR showed two separate ^{19}F signals for the bound ligand, which corresponded to two different conformational states for the complex. The 2D exchange spectrum (Figure 24) shows the two forms have exchange peaks with the free ligand but not with each other.[102] From intensity measurements, the ratio of the two populations A/B is 0.6/0.4 in the binary complex. In the ternary complex with NADP$^+$ the preference for binding is reversed with the A/B ratio becoming 0.3/0.7.[102]

We have used 2D exchange ^{1}H NMR spectroscopy to connect the signals from the bound species with their corresponding signals in the free ligand for complexes of DHFR formed with ligands **13**–**15** (spectra not shown). In the complex where the ligand has a symmetrically substituted phenyl ring, such as for **13** and **14**, four signals were observed for the four aromatic protons, which indicated that each is shielded differently. This result indicates the presence of hindered rotation about the phenyl–pyrimidine bond, with the phenyl ring taking up a fixed position within its binding

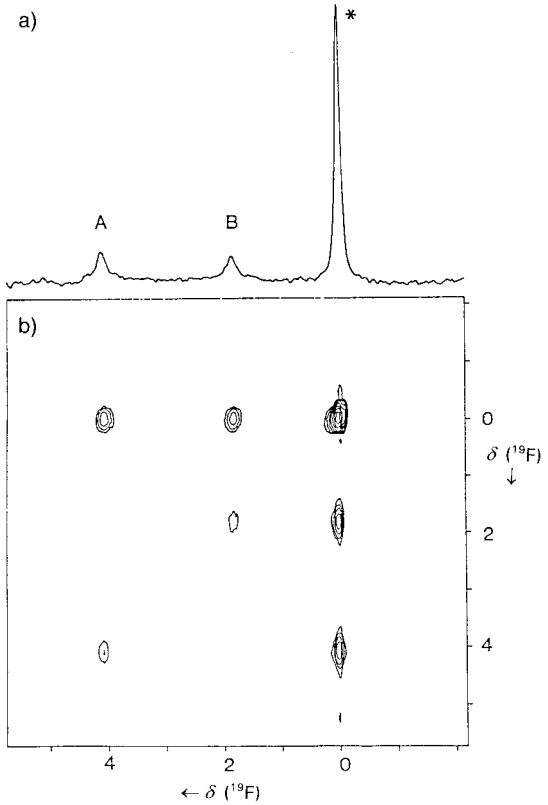


Figure 24. 376 MHz ^{19}F NMR spectra of 1.2 mM DHFR in the presence of excess 3′-nitro-4′-fluoropyrimethamine (**15**) at 308 K, pH 6.5. a) 1D spectrum, b) 2D NOESY exchange spectrum. Both spectra are referenced to the free ligand signal (indicated by an *). Forms A and B are seen to have exchange cross-peaks with the free ligand (magnetization transfer by exchange), but no exchange cross-peaks were observed between the Forms A and B. (Reprinted with permission from Tendler et al.[102])

site such that each of the four aromatic protons is in a different shielding environment in the protein. DHFR complexes containing pyrimethamine analogues with asymmetrically substituted phenyl rings such as **15** showed two complete sets of signals (three from Form A and three from Form B) for the phenyl protons in the bound ligands. Using X-ray data on related complexes[140, 165] together with NOE data on the *L. casei* DHFR complex it was possible to assign the ligand phenyl ring protons in bound pyrimethamine that are oriented towards Phe 30[102] and thus assign ligand signals to Forms A and B (Figure 25).

An understanding of the conformational preference for binding Form A in the binary complex of 3′-nitro-4′-fluoropyrimethamine (**15**) with DHFR can be reached by considering the model of the complex shown in Figure 25. In Form A the nitro substituent would be directed towards the vacant nicotinamide ring binding site and this could assist the favored binding. Addition of the NADP$^+$ to form the ternary complex reverses the preference for Form A, and this is consistent with the expected unfavorable steric interaction between the bulky nitro group and the nicotinamide ring of bound NADP$^+$.[102]

Ternary complexes of DHFR with pyrimethamine analogues and NADP$^+$ [102] were found to have the NADP$^+$ in two different conformations (Forms I and II),[156, 157] similar to those seen earlier for the DHFR-trimethoprim-NADP$^+$

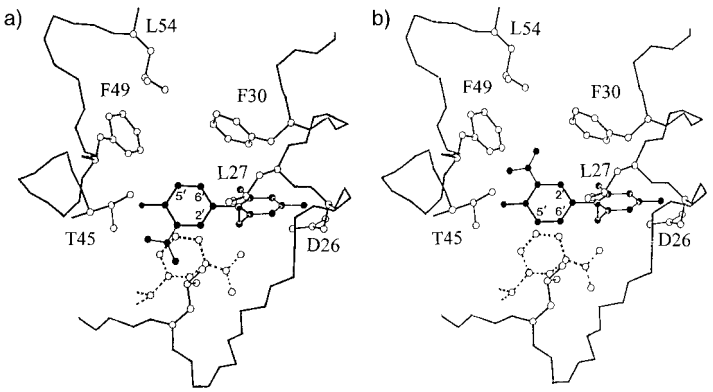


Figure 25. Model of the binding site in the 3′-nitro-4′-fluoropyrimethamine – DHFR complex for a) Form A and b) Form B. The position of the $NADP^+$ nicotinamide ring binding site is also indicated as a dotted line structure. The modeling used some crystallographic data from Matthews et al.[13, 14] (Reprinted with permission from Birdsall et al.[102])

complex. The results suggest that the two types of conformational states could be correlated with only Forms I B and II A being populated. This would be consistent with a structure having the nitro substituent oriented towards the vacant site for the nicotinamide ring binding in Form II A and oriented away from the nicotinamide ring binding site in Form I B (see Figure 25).

8.3. Complexes of *L. casei* DHFR with Folate and $NADP^+$

Complexes of *L. casei* DHFR with folate and $NADP^+$ provide further examples of ligands that bind in different conformational states to essentially the same binding site in the protein. The ternary complex of *L. casei* DHFR with folate and $NADP^+$ has been shown by NMR spectroscopy to have three distinct conformational states, designated Forms I, II a, and II b.[46, 90, 106, 157] Isotopically labeled (^3H, ^{13}C, and ^{15}N) folates were used in these NMR studies. For example, multiple ^{13}C signals corresponding to the three different forms have been seen in the ^{13}C spectrum of the complex of *L. casei* DHFR with $NADP^+$ and [4,6,8 a-^{13}C]folate (**16**) measured at various pH values[46] (Figure 26).

It can be seen that the populations of these conformational states are pH dependent. At pH 6.8 Forms II a and II b are present while at pH 5.5 the ternary complex is almost exclusively in Form I. The NOE measurements at pH 5.2 clearly indicate that the H7 proton of folate is close to the methyl group protons of Leu 19 and Leu 27 as shown in Figure 27. Similar NOEs were seen in a 2D HMQC – NOESY $^1H/^{13}C$ experiment on the complex with [4,7,8 a,9-^{13}C]methotrexate where edited NOEs between MTX H7 and the methyl protons of Leu 19 and 27 were detected.[46] Thus the orientation of the pterin ring in Form I is very similar to that of methotrexate in the DHFR – methotrexate complex. As mentioned earlier, this orientation is known to be about 180° from that of the corresponding ring in folate in its catalytically active complex (see Section 2).[46] NOE data from the DHFR-folate-$NADP^+$ complex indicated that Form II a

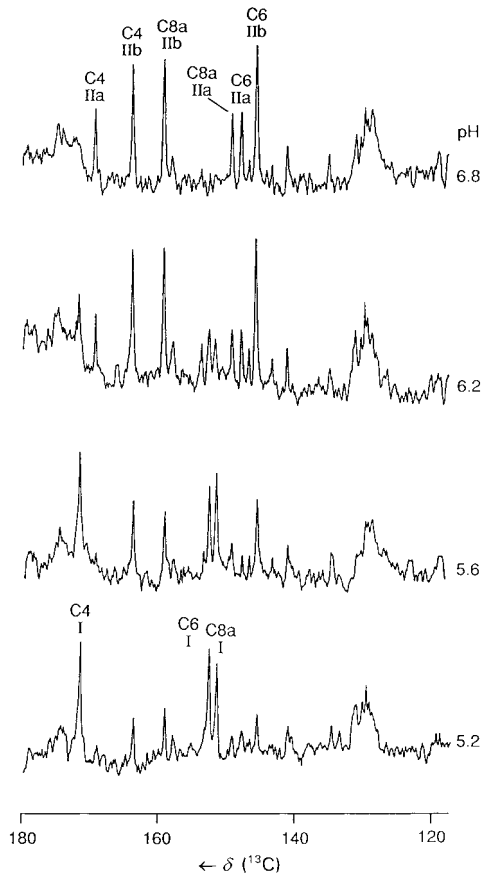
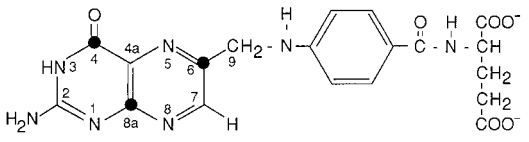


Figure 26. The low-field region of the 100.6 MHz ^{13}C NMR spectra at 281 K of the ternary complex of *L. casei* DHFR and $NADP^+$ and [4,6,8 a-^{13}C]folate at different pH values. Signals from the naturally occurring ^{13}C in the protein are also observed (at $\delta \sim$ ca. 130, 157, and ca. 175). (Reprinted with permission from Cheung et al.[46])

has a similar orientation to Form I (and to bound methotrexate (**5**)) but that Form II b has a different orientation of the pterin ring from that in the methotrexate complex. The H7 proton in Form II b shows an NOE connection to the Ala 97 methyl protons indicating its proximity to these protons but shows no NOEs to the Leu 19 and Leu 27 methyl protons (see Figure 27). These NOE results have been confirmed by examining the 2D $^1H/^{13}C$ HMQC – NOESY spectra of ^{13}C-labeled folates ([4,6,8 a-^{13}C]folate (**16**) and [2,4 a,7,9-^{13}C]folate (**17**)) bound to *L. casei* DHFR.[46]



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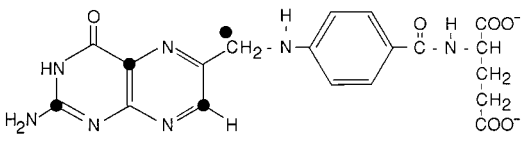

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From a consideration of the crystal structure data obtained by Bolin and co-workers[14] on the DHFR-MTX-NADPH complex it could be deduced that a "turned over" pteridine ring occupying essentially the same binding site would have its H7 proton close to the methyl protons of Ala97. Thus the major conformational difference between Forms I and IIb is the approximate 180° difference in orientation of the pterin ring in the two forms as illustrated in Figure 27. Form I (and Form IIa) has the pterin ring in the same orientation as the methotrexate complex, and Form IIb has the folate pterin ring turned over by 180° about an axis coincident with the ligand C2-NH_2 bond. In this orientation the catalytic reduction can proceed with the correct stereochemistry and this is referred to as the "active" conformation (Figure 27). In contrast, Forms I and IIa with the methotrexate-like orientation of the pterin ring correspond to "inactive" conformations (Figure 27). The folate pterin ring is seen to occupy approximately the same binding site in the different forms.[46, 157]

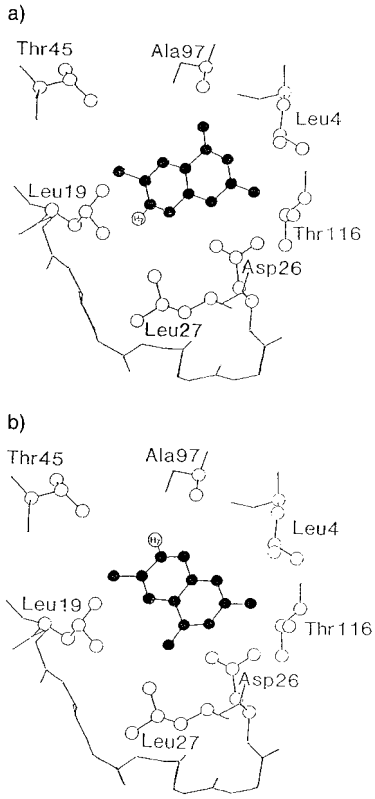
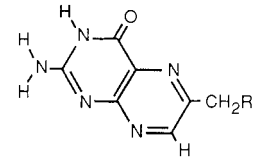
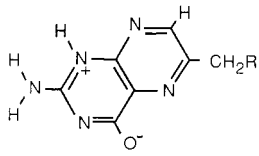


Figure 27. a) Proposed conformation of the pterin ring in the folate–DHFR complex in the "inactive" Forms I and IIa (based on the methotrexate conformation in the crystal structure data of Matthews and co-workers).[13, 14] b) Proposed conformation of the pterin ring in the folate–DHFR "active" Form IIb (turned over by about 180° compared to the other forms). (Reprinted with permission from Feeney.[24])

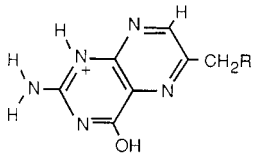
NMR studies of ^{13}C chemical shifts of bound folate have provided useful information about the tautomeric states in the three forms found in the complex with *L. casei* DHFR. By considering the ^{13}C chemical shifts of the labeled, bound folates **16** and **17** it was found that Form IIb is very similar to free folic acid at pH 5.5 for which the structure is known to be in the 4-keto form with N1 unprotonated[166] (see structure **18**).[46] This result gives no support to proposed enzyme mechanisms that require enolic forms as intermediates since only Form IIb has the "active" conformation of its pterin ring. In contrast, the C2 and C4 chemical shifts of bound folate in Form IIa are very different from those of free folic acid, which indicates a possible change in the keto/enol tautomerism or in the N1/N3 protonation states. Studies of model compounds provide estimates of the ^{13}C chemical shifts for various tautomeric and ionization states of folate. Comparisons of these with the experimental data for bound folate indicated that while Form IIb is in the keto form, Forms I and IIa exist as enolic forms (see structures **19** and **20**).



18 keto active form



19 enolic inactive forms 'methotrexate-like'



20

R = *p*-aminobenzoyl-L-glutamate

^{1}H NMR studies suggest that N1 is protonated in Forms I and IIa. This observation means that the tautomeric and ionization states in Forms I and IIa at N1 are both very similar to that in methotrexate which has a NH_2 group at the 4-position. Since the pterin ring has been shown to have the same orientation as in the methotrexate–DHFR complex, it is likely that the pterin ring in folate in Forms I and IIa bind to residues in the protein that are the same as those involved in methotrexate binding.

Site-directed mutagenesis studies involving Asp26 have provided direct evidence that this residue is involved in controlling the pH dependence of the conformational equilibrium.[167, 168] However, recent NMR studies of DHFR-folate-$NADP^+$ complexes using Asp-γ-$^{13}CO_2^-$-labeled DHFR and the mutant Asp26Asn DHFR have shown that the Asp26 residue influences the pH dependence of the equilibrium in an indirect manner by modifying the p*K* value of the pterin ring of bound folate.[51] This effect could result from Asp26 being close to the N1 position which is protonated in Forms I and IIa. The observation of multiconformational states in more than 20 different complexes of dihydrofolate reductase suggests that this phenomenon could also be common in other protein–ligand complexes. This is an important finding that has implications not only for understanding complex structure–function relationships but also for assisting in inhibitor design, since each different conformation provides a new starting point for exploring drug design.

9. Summary and Outlook

NMR spectroscopy has been shown to be a powerful technique for providing information about interactions, conformations, and dynamic processes in complexes of dihydrofolate reductase and antifolate drugs. It is likely that the increasing awareness of the capabilities of NMR methods for studying protein – ligand complexes and the wider accessibility of the high-field NMR spectrometers required for this type of work will encourage further research in this general area. The NMR experiments can now be executed with increasing facility and comparative studies of complexes of ligands with DHFRs from different species are becoming easier to carry out. This process should allow questions concerning the origins of ligand-binding specificity to different DHFRs (for example bacterial and mammalian) to be addressed more directly. Structural studies in solution on complexes of target DHFRs from species not yet examined (such as *Plasmodium falciparum* and *P. carinii*) will no doubt be carried out.

Improved methods of structure determination—for example by incorporating chemical shift information—will eventually provide more detailed information about protein – ligand complexes in solution. The recently developed approaches for obtaining structural information using dipolar coupling contributions that result from orienting the molecules in solution (either by using high magnetic field, liquid crystal or phage orienting approaches) should have an important impact on structural studies of DHFR – ligand complexes.[169] The improved structures should allow the detection of smaller differences in structure between different complexes. Future work should also lead to an improved understanding of the dynamic processes taking place within ligand – protein complexes.

Fesik and co-workers[170, 171] have suggested a novel method for designing tightly binding enzyme inhibitors that could be applied to DHFR. In this method large numbers of ligands are quickly screened for their potential binding to a target protein by detecting changes in chemical shift in $^{1}H/^{15}N$ HSQC spectra of the ^{15}N-labeled protein in the presence of batches of ligands. A binding ligand is identified as one that perturbs the protein $^{1}H/^{15}N$ chemical shifts. Once such a binding ligand has been identified, the protein is saturated with this ligand, and the screening continued to find another ligand that binds noncompetitively with the first one. When a suitable second candidate is found, detailed NMR structural work on the ternary complex is undertaken and—on the basis of the structural information obtained—a strategy is developed for chemically linking the weakly binding ligands to produce a high affinity binding ligand. This approach has been used to produce inhibitors with high binding affinity for metalloproteinases such as stromelysin.[171] A similar approach could be adopted to find new lead compounds as potential inhibitors of dihydrofolate reductase. Such work would best be carried out in industrial laboratories where suitable libraries of compounds are readily available for the screening.

It is clear that DHFR, in addition to its intrinsic pharmacological interest, has proved to be an excellent test-bed for exploring ideas and methods of tackling the problem of binding specificity and many of the results obtained on this system will have general applicability.

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