Biochemical Pharmacology, Vol. 40, No. 1, pp. 141–152, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

NMR STUDIES OF INTERACTIONS OF LIGANDS WITH DIHYDROFOLATE REDUCTASE

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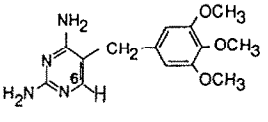
Abstract—NMR spectroscopy is a useful technique for studying interactions, conformations and dynamic processes within ligand–protein complexes. Several examples of the application of the method to studies of complexes of anti-folate drugs with their target enzyme, dihydrofolate reductase, are discussed.

Dihydrofolate reductase (DHFR*) catalyses the reduction of dihydrofolate (or folate, with lower efficiency) to tetrahydrofolate using NADPH as coenzyme.

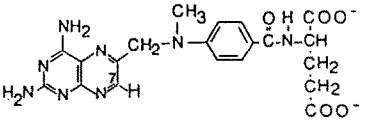
Folate + NADPH + H^+
$\rightleftharpoons$ 7,8-dihydrofolate + $NADP^+$
7,8-Dihydrofolate + NADPH + H^+
$\rightleftharpoons$ 5,6,7,8-tetrahydrofolate + $NADP^+$

DHFR is an essential enzyme in all cells since the final product of the reduction, tetrahydrofolate, is a required cofactor in a number of biosynthetic processes involved in the synthesis of purines, pyrimidines and some amino acids [1]. The considerable pharmacological interest in the enzyme derives from the fact that it is the target for several clinically-used anti-folate drugs such as trimethoprim, **I** (antibacterial), and methotrexate, **II** (antineoplastic).

The historical perspectives on this important enzyme have been reviewed recently in the 1988 Nobel Lecture in Physiology or Medicine by Dr. G. H. Hitchings [2]. In the search for improved drugs, many methotrexate and trimethoprim analogues have been synthesised and evaluated since the original synthesis of the parent compound (Seeger *et al.* [3]; see also review by Roth and Cheng [4]). As the structural characterisation of the enzyme has progressed, there has been an increasing interest in trying to understand the factors controlling the origins of the specificity of binding (such as why trimethoprim binds much more tightly to bacterial DHFR compared with the mammalian enzyme). One approach to trying to characterise individual interactions has been to compare the binding energies before and after a structural modification of ligands or protein aimed at removing or introducing a specific interaction. The advent of site-directed mutagenesis methods has obviously widened the scope for such experiments. However, in assessing contributions from specific interactions in this way it is not sufficient simply to measure the binding energies; it is essential that such measurements should also be accompanied by some structural measurements aimed at assessing whether the particular interaction has been influenced in the manner predicted and whether or not any conformational changes or additional perturbations have been introduced. Extensive X-ray [5–9] and NMR [10–74] investigations of the structures and interactions in complexes formed by DHFR with various inhibitors have been reported. While X-ray crystallography gives a precise description of the structure of the complex in the crystal state, NMR has the advantage that it examines the complexes in solution and



Trimethoprim **I**



Methotrexate **II**

Scheme 1

* Abbreviations: DHFR, dihydrofolate reductase; NOE, nuclear Overhauser effect; COSY, correlated spectroscopy; RELAY, relayed correlated spectroscopy; and HOHAHA, homonuclear Hartmann–Hahn spectroscopy.

does not require crystals. However, the availability of X-ray structural data on some complexes is often crucial for achieving a detailed interpretation of the NMR data obtained either on the same or on related complexes for which no X-ray structural

data are yet available. In addition to the studies aimed at understanding the specificity of ligand binding, there have been intense efforts in several laboratories (notably those of Blakely [15], Kraut [75, 76] and Benkovic [77, 78]) to unravel the details of the enzyme mechanism which is still not understood completely.

In our laboratory we have been examining the NMR spectra of complexes of the *Lactobacillus casei* DHFR with various substrates and inhibitors in order to obtain detailed information about the protein–ligand binding in the complexes [10, 14, 16–40, 42–56, 58–67, 70–74]. Such investigations can contribute to our understanding of both binding specificity and catalytic mechanisms in several ways:

(i) by providing information about individual interactions between groups on the ligand and on the protein,
(ii) by characterising the conformational changes in the ligand and protein which accompany ligand binding,
(iii) by measuring conformational equilibria in complexes where multiconformational forms are present, and
(iv) by defining changes in intra- and inter-molecular dynamic processes observed on complex formation (such as rates of ring-flipping in bound inhibitors such as methotrexate [13, 42, 43] and trimethoprim [13, 43] and rates of hydrogen-bond breaking in the trimethoprim · DHFR complex [14, 43]).

To extract this information from the NMR spectra it is necessary first to assign NMR signals to specific nuclei in the ligand or protein.

Assignment of ligand signals

For weakly binding ligands which show fast exchange behaviour on the NMR chemical shift time scale, there is no problem with signal assignment. As increasing amounts of the ligand are added to the protein solution, the ligand signals (which are averaged signals from the free and bound ligands) appear in the spectrum with increasing intensities and shift progressively towards the chemical shifts of the free ligand. A detailed analysis of the binding curve obtained by plotting the observed averaged chemical shifts for the ligand signal against the ligand concentration yields the bound and free chemical shifts and the binding constant. NMR signals from nuclei in fragments of methotrexate and trimethoprim such as *p*-aminobenzoyl-L-glutamic acid and 2,4-diaminopyrimidine ($K_d \sim 10^3$–10^4 M) are usually in fast exchange [10–12].

For tightly bound ligands such as methotrexate and trimethoprim, slow exchange behaviour is observed and separate signals are detected for free and bound ligands. In these cases the assignments can be made directly by using analogues containing selective isotopic substituents (^{2}H, ^{13}C or ^{15}N): the differences in the NMR spectra of the complexes formed with normal and isotopically labelled ligands can be used to locate the positions of the bound ligand signals [13, 17].

Transfer of saturation measurements can sometimes be used to connect bound and free ligand signals in spectra from complexes showing slow exchange behaviour if they have sufficiently rapid dissociation rates ($>1\ \text{sec}^{-1}$) to allow the transfer of saturation to compete favourably with relaxation mechanisms [17–23]. In such cases there is also the opportunity to carry out transferred NOE experiments to provide information about the bound ligand conformations from NOE observations made on the free ligand signals [24, 25].

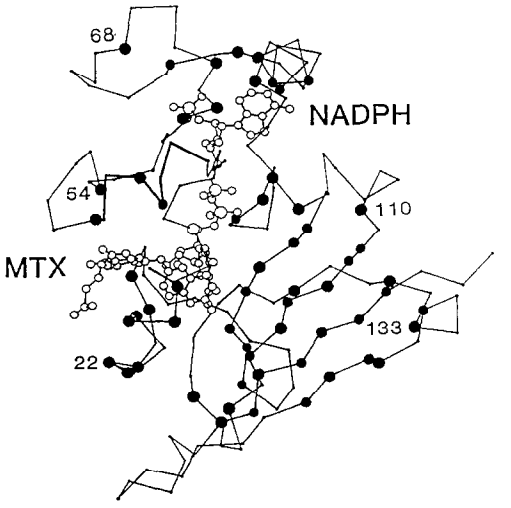


Fig. 1. X-ray crystal structure of DHFR · methotrexate · NADPH complex (from the data of Matthews and coworkers) showing the α-carbon backbone of the enzyme and the non-hydrogen atoms of the ligands. Residues for which proton resonances have been assigned are indicated by solid black circles.

Studies of bound ligand signals have proven to be particularly rewarding in monitoring ionisation states [13, 14, 26–29], multiple conformations [30–41, 65] and dynamic processes within the binding site of the complex [13–14, 17, 42–44].

Assignments of protein signals

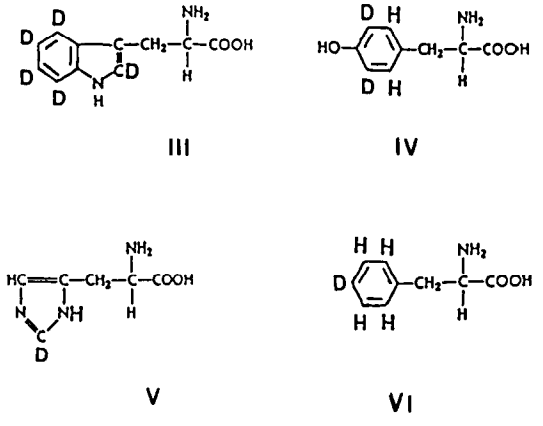
Assignments of ^{1}H signals in the spectra of *L. casei* DHFR have now been made for 59 of the 162 amino acid residues (see Fig. 1). For proteins of this size ($M_r \sim 18$K), it is usually necessary to resort to isotopic substitution methods in order to assist the assignment procedure. In these cases the complexity of the spectra and the degeneracy of the chemical shifts make it very difficult to apply the direct sequential assignment procedures of the type pioneered by Wüthrich and coworkers (see Wüthrich [79] and references therein).

The assignment method we have used is a two-stage process. First, we assign the signals to their residue types by using a combination of selective deuteration and 2D experiments such as COSY, RELAY and HOHAHA. Second, we use NOE measurements to identify signals from protons in different residues which are close together and then correlate these with protons which are predicted to be in close proximity from the crystal structure data (the method assumes that the crystal and solution

structures are not too different). Selectively deuterated amino acids can be incorporated biosynthetically into *L. casei* (which is auxotrophic for all the amino acids) and the dihydrofolate reductase then isolated and purified using procedures previously developed for the non-deuterated enzymes [45–55]. In this way we have produced four selectively deuterated proteins with the following composition:

(i) DHFR with all valine γ-Me protons replaced by deuterium [49, 50],
(ii) DHFR with all aromatic protons except Trp 2′-protons replaced by deuterium [48],
(iii) DHFR with all aromatic protons except Tyr 2′,6′-protons (or 3′,5′-protons) replaced by deuterium [45–47], and
(iv) DHFR with His 2′-protons, Trp 2′,4′,5′,6′,7′-protons, Tyr 3′,5′ protons and Phe 4′-protons replaced by deuterium (see Scheme 2) [51].

Ideally one should aim for a pattern of selective deuteration which is sufficient to remove some of the chemical shift ambiguities but which retains enough protons to allow the necessary NOE connections to be made for use in the assignment to specific residues. This is exemplified by the deuterated DHFR prepared by incorporating the aromatic amino acids shown in Scheme 2 (protein **IV**). This pattern of deuteration was selected such that only Phe residues will give rise to cross-peaks in the aromatic region of the COSY spectra:



Scheme 2

the resulting simplification can be seen by comparing the COSY spectra in Fig. 2a and 2b. In the spectrum from the complex formed with the selectively deuterated enzyme and methotrexate (Fig. 2b), the cross-peaks from 7 of the 8 Phe residues can be identified immediately. The signals for the remaining Phe residue proved to be a strongly coupled "AB" multiplet which could be detected by examining the one-dimensional spectrum of the complex [51].

To make sequence specific assignments of the Phe signals, it is necessary to correlate the NOE connectivities between the Phe protons and other protons in the protein with internuclear distance information obtained from the crystal structure. The selective deuteration simplifies not only the COSY spectrum but also the NOESY spectrum as shown in Fig. 3 (which compares the NOESY spectra for methotrexate complexes formed with the normal and deuterated enzyme). This simplification clearly assists in helping to make assignments by removing ambiguities about the origins of the NOESY cross-peaks. A good example of this is seen by comparing the cross-peaks in row F_1 (−4.71 ppm) in Fig. 3 which correspond to several aromatic signals with NOE connections to the previously assigned methyl proton signal of Leu 113: in the spectrum from the complex formed with the deuterated enzyme only two NOE cross-peaks are observed in row F_1 −4.71 ppm. Examination of the crystal structure data indicates that Phe 136 is the only Phe residue which is sufficiently close to Leu 113 to give these effects; this allows us to assign the corresponding signals to Phe 136. Using this approach, signals from the 8 Phe residues together with all the other aromatic amino acid residues in DHFR have now been assigned (Birdsall *et al.* [81]). The use of such deuterated proteins from organisms grown on carefully selected deuterated amino acids is becoming a routine approach for assisting in the assignment procedure in NMR spectra of large proteins.

The assigned signals have been used as reporter groups to provide information on conformational changes in several complexes formed by substrate analogues binding to wild-type and mutant dihydrofolate reductases [56].

Measuring ionisation states and comparison of protein–ligand structures

NMR has proven to be an ideal method for measuring ligand and protein ionisation states in complexes of dihydrofolate reductase with substrates and inhibitors. ^{13}C and ^{15}N labelled substrate analogues are particularly useful for investigating ionisation states in the ligand. For example, measurements of ^{13}C chemical shifts of bound [^{13}C-2]methotrexate (Cocco *et al.* [57]) have shown that the N1 position of bound methotrexate is protonated in its complex with the enzyme. By examining complexes of the enzyme with [^{13}C-2]trimethoprim we have similarly shown that the N1 of bound trimethoprim is also protonated in its bound state [16]. In this case the signal for the NH proton can be detected directly in ^1H spectra by examining enzyme complexes formed with ^{15}N-1-trimethoprim in H_2O solution [14, 15].

Ionisation states of the protein His residues have also been measured [58, 61]. In earlier studies we showed that the pK of His 28 is increased by ~1 unit when its charged imidazole ring interacts with the γ-CO_2^- of the glutamate moiety of methotrexate (or indeed of any folate analogue): NMR can be used to monitor this pK in complexes formed with methotrexate analogues and thus it is possible to detect whether or not the interaction between the γ-CO_2^- and His 28 is taking place (Fig. 4). NMR can also be used for demonstrating similarities and differences in the mode of binding of various substrate analogues to the enzyme, and the above methods of monitoring

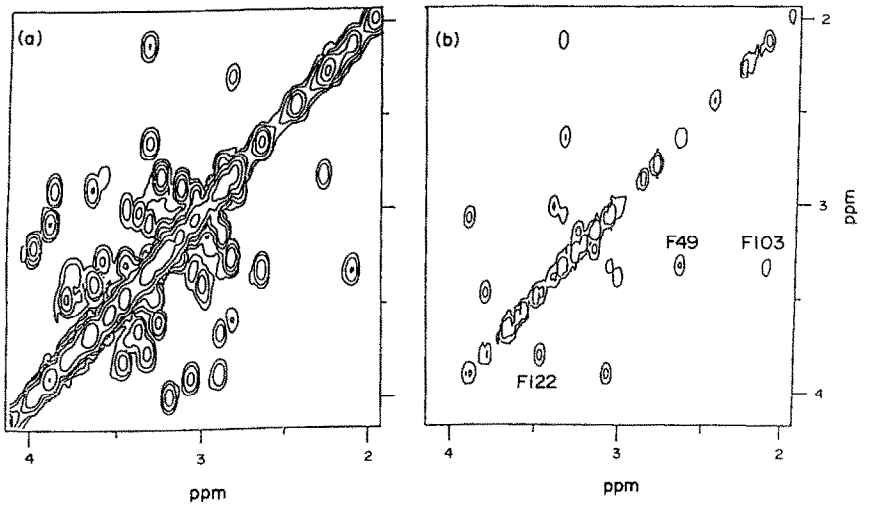
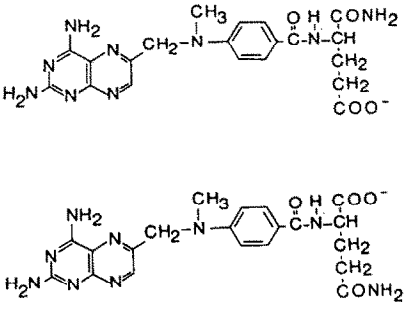


Fig. 2. Aromatic region of the 2D COSY ^{1}H contour plot for the DHFR · methotrexate complex recorded at 500 MHz. (a) Non-deuterated enzyme; and (b) selectively deuterated enzyme incorporating the selectively deuterated amino acids (**III–VI**). The ordinates are the same on the left and the right, and the chemical shifts are measured with reference to dioxan. Reprinted with permission from *FEBS Lett* **248**: 57–61, 1989. [Ref. 51].

ionisation states in the complexes provide a powerful tool for such studies. This approach has proven particularly useful for studying the enzyme complexes formed with the α- and γ-amide methotrexate analogues (structures **VII** and **VIII** respectively, Scheme 3) [62].

Methotrexate Amides



Scheme 3

These structural modifications of the glutamate moiety influence the binding in different ways: the γ-amide analogue binds an order of magnitude less tightly than methotrexate, whereas the α-amide binds 100 times more weakly. For the DHFR complex formed with the γ-amide analogue, the pH titration of the His 28 C2 proton chemical shifts indicated that the pK of His 28 was not perturbed from its value in enzyme alone but was ~1 pK unit different from its pK value in the enzyme · methotrexate complex. An examination of the 2D COSY spectra [63] revealed that the ^{1}H signals

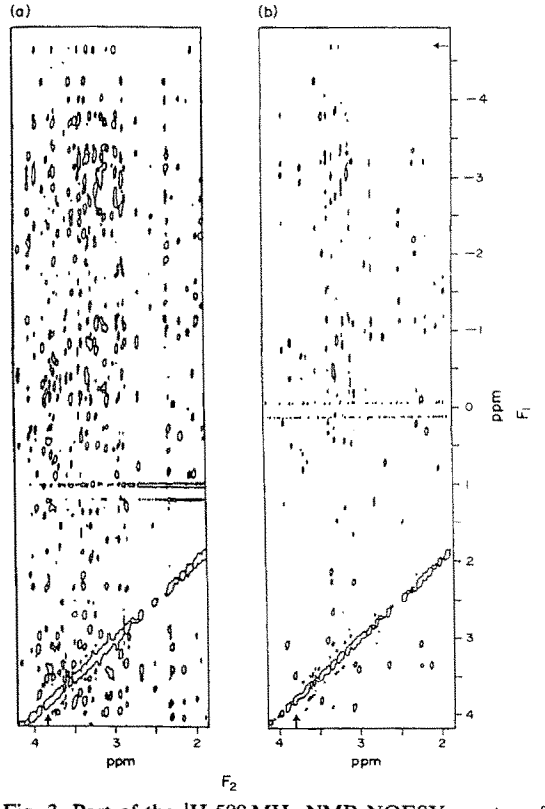


Fig. 3. Part of the ^{1}H 500 MHz NMR NOESY spectra of the DHFR · methotrexate complex obtained (a) with non-deuterated enzyme, and (b) with the deuterated enzyme sample. Reprinted with permission from *FEBS Lett* **248**: 57–61, 1989. [Ref. 51].

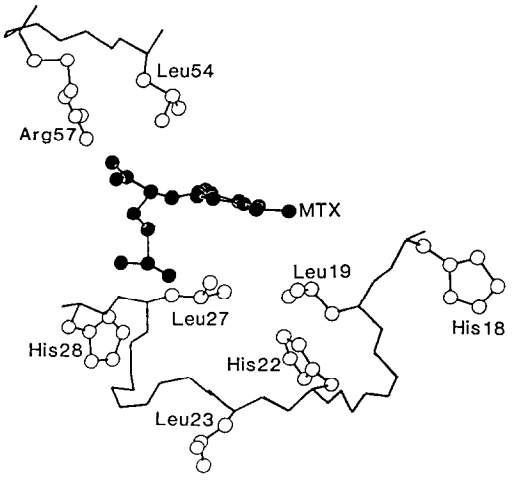


Fig. 4. Part of the crystal structure of the DHFR·methotrexate·NADPH complex (from the data of Matthews and coworkers) showing residues around the methotrexate binding site.

of assigned residues in the protein have almost identical shifts to those of corresponding protons in the methotrexate·enzyme complex for all residues except His 28 and, to a lesser extent, Leu 27 (see Fig. 5b). This indicates that while the His28 interaction with the MTX γ-CO_2^- is no longer present in the complex with the γ-amide, there has not been a major change in the overall structure of the two complexes. This behaviour contrasted with that seen for the α-amide complex where the shifts of protons in several protein residues were different from their values in the complex formed with methotrexate (see Fig. 5a). Although the γ-carboxylate has not been modified, the pK of His 28 is again ~1 unit lower than its value in the enzyme·methotrexate complex and similar to its value in the enzyme alone. This clearly indicates that the modification of the α-carboxylate is not only removing the α-carboxylate interaction with Arg 57 (see Fig. 4) but is also preventing the γ-carboxylate from interacting with His 28. The removal of both carboxylate interactions explains why the α-amide binds an order of magnitude less tightly to the enzyme than does the γ-amide analogue where only the γ-carboxylate–His 28 interaction is removed. The other residues in the protein whose protons are most affected are all near to the benzoyl ring of methotrexate α-amide (such as Leu 27, Phe 49, Leu 54 and Leu 19), and their shieldings are expected to be influenced by ring current contributions from the benzoyl ring. The observed shift differences indicate that the previously discussed disruption of the α-CO_2^- interaction with Arg 57 is accompanied not only by the breaking of the γ-CO_2^- interaction with His 28 but also by a change in orientation of the benzoyl ring [62, 63].

Clearly NMR can provide a convenient method of demonstrating structural variations in complexes with these different methotrexate analogues.

Rationally designed trimethoprim analogues

From a comparison of the crystal structures of the trimethoprim and methotrexate complexes of the *Escherichia coli* enzyme [9] and from NMR studies in the complexes with the *L. casei* enzyme [17–23], it can be deduced that the 2,4-diaminopyrimidine ring of trimethoprim binds in the same binding site as the corresponding part of the pteridine ring of methotrexate. When we consider the model for trimethoprim [or brodimoprim (**IX**)] in its complex with the *L. casei* enzyme, it is clear that the trimethoprim, unlike methotrexate, cannot make any direct interactions with Arg 57 and His 28 (see Fig. 6). In collaboration with colleagues from Hoffmann-La-Roche [64], we have prepared and studied several analogues of brodimoprim (**IX**) which were designed to have improved binding to the enzyme by making additional interactions with Arg 57 and/or His 28. Computer graphics techniques were used to assist in designing side-chains at the 3′-O-position of brodimoprim aimed at making these interactions (see Table 1).

Table 1 contains the measured inhibition constants for the various analogues and provides some estimate of their improved binding. NMR was used to monitor the pK values of His 28 residues in their complexes with DHFR and these measurements allow us to assess if the side-chains are binding in the predicted manner. The 4,6-dicarboxylate analogue (**XII**) binds 3 orders of magnitude more tightly to DHFR than does the parent molecule and provides a ~1 unit pK shift in the pK of His 28, clearly indicating that the carboxylate–His 28 (see Fig. 7) interaction has been made. However, the 6-monocarboxylate analogue (**XI**) which is modelled to achieve the carboxylate–His 28 interaction does not in fact perturb the pK value of His 28 even though it binds fairly tightly. Although the 6-carboxylate analogue can reach His 28, it appears to prefer to bind at some alternative site, possibly Arg 57. It is clear that in favourable cases NMR offers a convenient manner of assessing when predicted interactions are taking place in complexes formed with rationally designed inhibitors [64].

A similar study aimed at designing tightly binding analogues of trimethoprim has been undertaken by Kuyper and coworkers [9]: in this case X-ray crystallography was used to determine their mode of binding.

Multiple conformations in complexes of substrates with dihydrofolate reductase

Although the substrate folate has a fairly similar structure to methotrexate it has been found that the orientation of the pteridine ring in the catalytically active form of the substrate–enzyme complex is "turned over" by ~180° compared with that in the methotrexate·enzyme crystal structure [68–72]. More recently, NMR studies on the enzyme·folate and enzyme·folate·$NADP^+$ complexes have shown that these exist as mixtures of two or more slowly interconverting conformations in solution [30–33]. For example the enzyme·folate·$NADP^+$ complex exists as a mixture of three conformations (forms I, IIa and IIb) whose proportions are pH dependent [30, 31]. Resonances have now been assigned for all the aromatic protons in the bound ligands in the different conformational states, and it is clear from

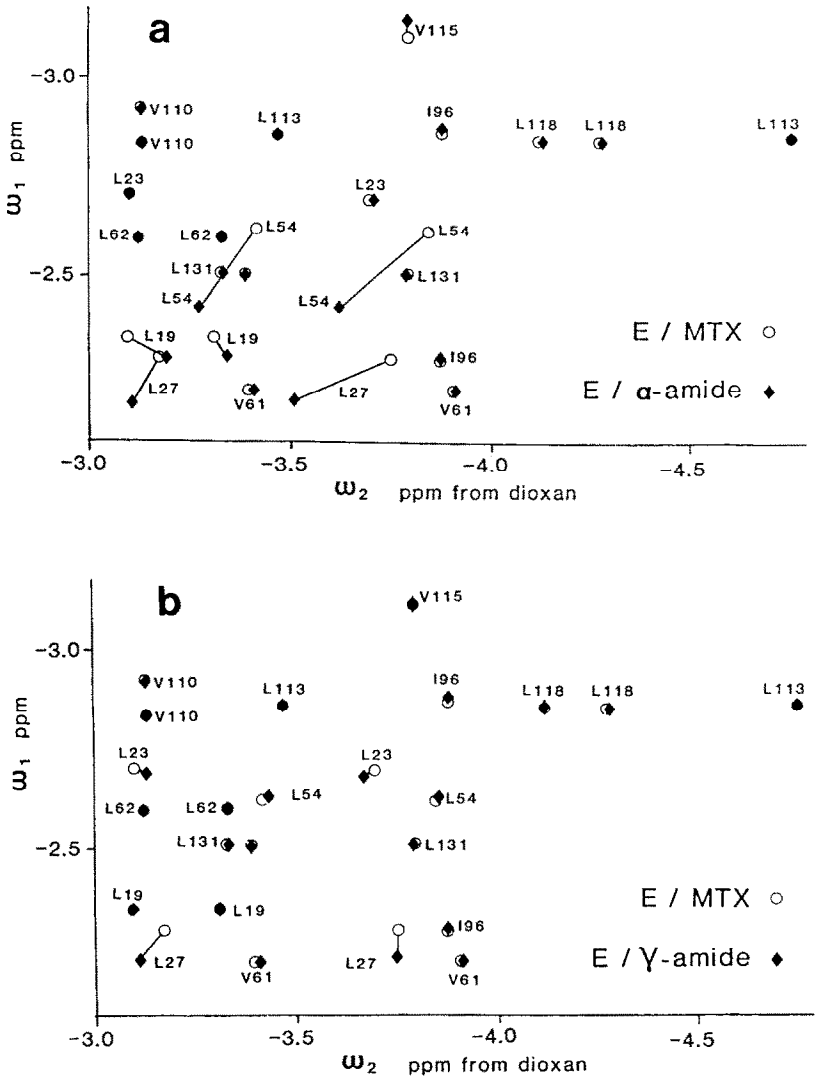


Fig. 5. Schematic representation of the aliphatic region of the 2D COSY ^{1}H contour plots for DHFR·methotrexate and (a) DHFR·methotrexate α-amide and (b) DHFR·methotrexate γ-amide. Reprinted with permission from *Biochemistry* **26**: 8585–8590, 1987. Copyright (1987) American Chemical Society. [Ref. 63].

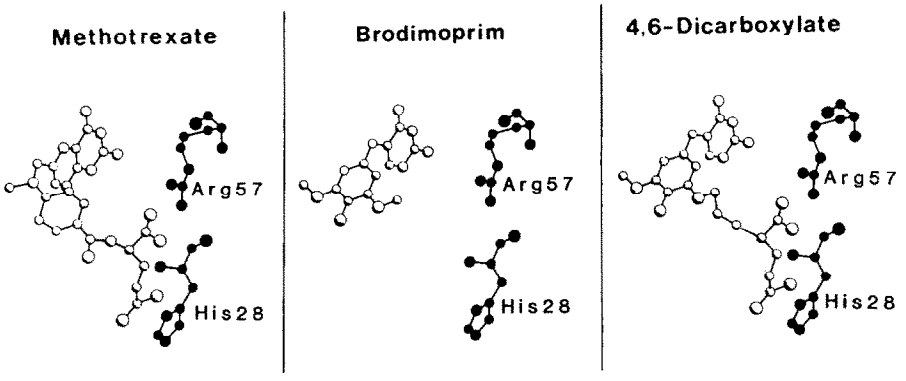
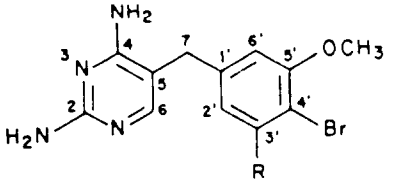
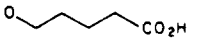
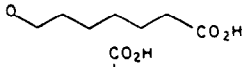
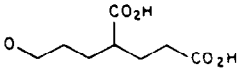


Fig. 6. Conformations of methotrexate, brodimoprim, and the 4,6-dicarboxylate analogue (**XII**) in their complexes with DHFR [64].

Table 1. Inhibition constants (K_i) and His-28 pK values for complexes of *L. casei* dihydrofolate reductase with brodimoprim and its derivatives (**IX–XII**)



Compound	R	K_i (nM)	pK His-28
IX	OCH_3	11.3	6.80
X		0.2	6.83
XI		0.6	6.80
XII		<0.01	7.80
Methotrexate		0.004	7.80

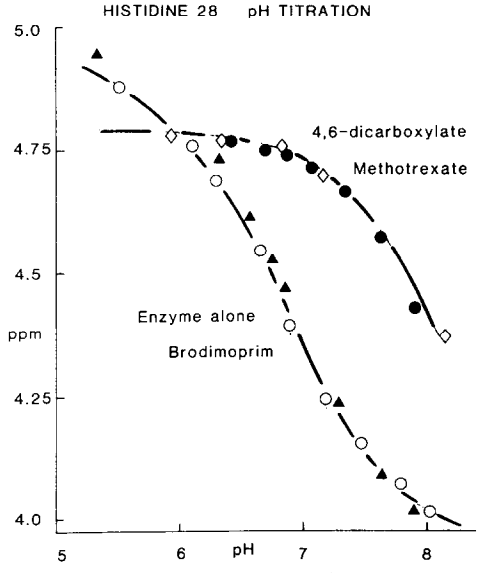


Fig. 7. The pH titration curves of the ^1H chemical shifts of the C-2 protons of the His 28 residue in DHFR (○) and its complexes with brodimoprim (▲), methotrexate (◇) and the 4,6-dicarboxylate analogue (**XII**) (●). (Birdsall, Feeney and Roberts, unpublished data; and Ref. [64].)

the observed chemical shift differences for protons in the ligand and protein that the structural differences between the different conformations are localised in the binding site of the enzyme. The COSY spectra of the protein in all three forms are very similar to that observed for the enzyme · methotrexate · $NADP^+$ complex, indicating that there can only be small differences in protein conformation in the various complexes. The NOE enhancements between the nicotinamide H5 and H6 protons and the Thr 45 methyl protons are similar for the complexes at both low and high pH, indicating that there are no major conformational differences involving the nicotinamide ring between the different forms. The largest chemical shift differences are seen for the pteridine ring H7 proton of folate which varies by more than 1 ppm in the different forms. In the low pH form (form I) and in form IIa (but not IIb) an NOE connection is observed between the H7 of bound folate and the methyl protons of Leu 19 and Leu 27. Similar NOE connections have been detected in the enzyme · methotrexate · NADPH complex where the pteridine H7-proton is known to be less than 4 Å from the methyl protons of Leu 19 and Leu 27 in the crystal structure [33]. In form IIb, no NOE connections could be detected between the folate H7 and the Leu methyl protons. One model which would satisfy all the observations would have forms I and IIa with the pteridine ring orientation similar to that in the methotrexate complex while form IIb would have the folate pteridine ring turned over by 180° about an axis approximately coincident with the C2—NH_2 bond. Such an orientation would allow the catalytic reduction to proceed with the correct stereochemistry and can be considered as the "productive" conformation (Fig. 8b). Forms I and IIa with the methotrexate-like orientation of the pteridine ring would correspond to "non-productive" conformations (Fig. 8a).

The ionisable group responsible for the pH dependence of the equilibrium between the three forms has been estimated to have a $pK < 5$ in states IIa and IIb and $pK > 7$ in state **I** [32]. We will see later that site-directed mutagenesis studies involving Asp 26 have provided direct evidence that this is the residue involved in controlling the pH dependence of the conformational equilibrium [73].

Complexes formed with mutant dihydrofolate reductases

Several mutant *L. casei* dihydrofolate reductases have been prepared (using site-directed mutagenesis methods), and their kinetic behaviour, ligand binding properties and structures have been compared with those of the wild-type enzyme [15, 73–79].

Asp 26 → *Glu and Asp* 26 → *Asn DHFR mutants.*

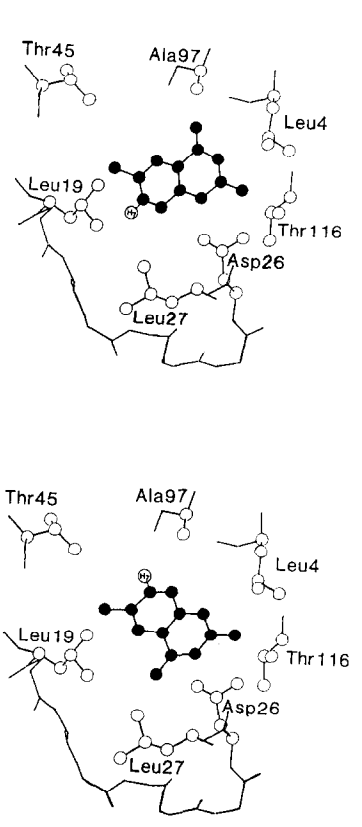


Fig. 8. (a) Conformation of the methotrexate pteridine ring in its binding site in DHFR (from the data of Matthews and coworkers). (b) Proposed conformation of pteridine ring in the "productive" conformation of the folate · DHFR complex.

A good deal of attention has been given to site-directed mutants involving substitution of Asp 26 because of its potentially important role both in the catalytic process and in inhibitor binding [34, 73–77, 79].

The substitution of Asp 26 by Glu does not cause a major perturbation in trimethoprim binding showing that the sequence difference at residue 26 which is a conserved Asp in bacterial enzymes but a Glu residue in vertebrate enzymes [8] does not in itself confer the specificity of binding of trimethoprim to bacterial over vertebrate enzymes [74]. Although the bound chemical shifts of the trimethoprim in its complex with the Asp 26 → Glu enzyme indicate no large changes in the overall bound conformation, the mutation is sufficient to perturb some of the dynamic processes within the complex. Thus, the rate of breaking and reforming of the hydrogen bond between the trimethoprim N1 proton and the carboxylate group at residue 26 is increased by an order of magnitude in the complex with the Asp 26 → Glu mutant. Furthermore, the rate of ring-flipping of the trimethoprim benzyl ring is four times faster in the Asp 26 → Glu complex. Since it is likely that transient conformational rearrangements of the protein structure are required to allow these dynamic processes to proceed, these conformational fluctuations in the protein must be influenced in a sensitive manner by the structural differences between the mutant and wild-type enzyme.

We have already seen that the complex formed by folate and $NADP^+$ with the wild-type enzyme exists as a mixture of three conformational forms (I, IIa and IIb). The complex formed with the Asp 26 → Glu enzyme also exists in the same forms which show essentially the same pH dependence with respect to the ratios of the different forms. However, for the folate · $NADP^+$ complex formed with the Asp 26 → Asn mutant very different behaviour is observed. In this case essentially only one form (>90%) was detected over the pH range 5.0 to 7.1 and this corresponds to form I, the "methotrexate-like" conformation. This provides strong evidence that Asp 26 is the residue controlling the pH dependence of the equilibrium in the complex formed with the wild-type enzyme [73].

Several groups of workers have shown that the Asp 26 → Asn mutation (Asp 27 → Asn for *E. coli*) results in very low catalytic activity [15, 75]. This has been taken as strong evidence that Asp 26 is either directly or indirectly involved in assisting the initial proton donation to N5 of 7,8-dihydrofolate. However, one cannot exclude the possibility that the Asp 26 → Asn mutation could result in an unfavourable unproductive conformation of the substrate which would result in low catalytic activity. Studies of the enzyme complexes formed with 7,8-dihydrofolate and $NADP^+$ to see if such forms exist have not, as yet, been undertaken.

Trp 21 → *Leu mutant.* Replacement of the conserved Trp 21 by a Leu residue causes a large decrease in enzyme activity and a reduction in NADPH binding by a factor of 400 but has little effect on the binding of substrates and substrate analogues (less than a factor of 3) [74]. A detailed comparison of the kinetic behaviour of the mutant and wild-type enzymes has been undertaken in Benkovic's laboratory [77]; this revealed that the Leu substitution results in a decrease in the rate constant for hydride transfer by a factor of 100 making it the rate-limiting step in the steady-state turnover.

In the aliphatic regions of the 2D COSY spectra of the complexes of methotrexate with the mutant and wild-type enzyme (Fig. 9), several assigned signals for particular protons have different chemical shifts. Some of these are for protons in close proximity to the aromatic rings of Trp 21 and these have different shifts in the mutant enzyme compared with the wild type as a result of removal of the Trp ring current effects: these include resonances from Leu 118, Leu 19 and Leu 23. There is a second group of shifted resonances which arises from protons remote from the Trp 21 (up to 14 Å from the site of substitution): for example, Leu 27, Leu 54, Leu 4, Val 110, and Val 115 all have protons that shift by more than 0.05 ppm between the two complexes. While it is not possible to give a quantitative explanation of the shifts, a consideration of the shielding gradient factors [63] indicates that they could result from fairly small changes in conformation (<0.2 Å).

Measurements of the ^1H chemical shifts in bound

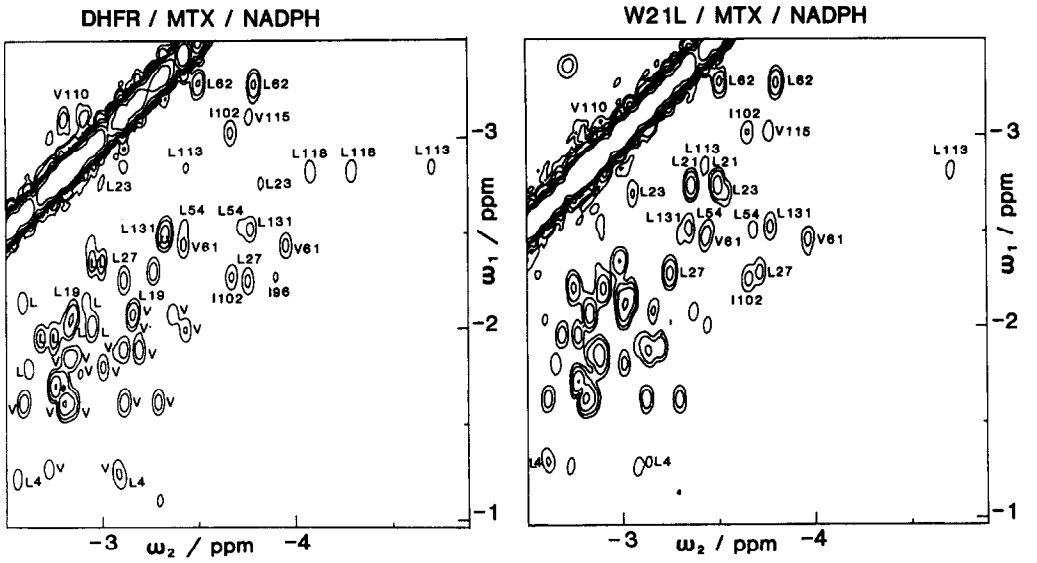


Fig. 9. Aliphatic regions of the 2D COSY ^{1}H contour plots for the DHFR·methotrexate·NADPH complex using wild-type enzyme and Trp 21 → Leu DHFR. Reprinted with permission from *Biochemistry* **28**: 1353–1362, 1989. Copyright (1989) American Chemical Society. [Ref. 74].

NADPH in the mutant and wild-type enzyme complexes indicate that the reduced nicotinamide ring is binding differently in the two complexes [74]. In contrast, ^{13}C and ^{1}H chemical shift measurements for nuclei in bound substrate and substrate analogues show that these bind similarly to the mutant and wild-type enzymes. The crystal structure data of Matthews and coworkers [6] indicate that the C ζ2 proton of Trp 21 has hydrophobic interactions with the carboxamide nitrogen of bound NADPH. Clearly the substitution of Trp 21 by Leu leads to loss of this interaction and to a change in orientation of the nicotinamide ring within the binding site. The reduced nicotinamide ring is known to contribute a large amount to the overall binding energy of NADPH, and a perturbation of its interaction with the enzyme could account for the 400-fold reduction in coenzyme binding. It could also account for the large decrease in catalytic activity. We previously observed a similar diminution in coenzyme binding and catalytic activity in experiments using thio-NADPH instead of the normal coenzyme [77, 80].

The Trp 21 → Leu substitution also perturbs some of the delicately poised equilibria involving multiple conformations observed for some complexes. In complexes of the wild-type enzyme with trimethoprim and $NADP^+$ there is a mixture of two almost equally populated conformations (forms I and II) each giving separate NMR spectra [36–40]. In the corresponding complex with the mutant enzyme only one of the conformations (form II) is detected: in this form the nicotinamide ring of $NADP^+$ is extended away from the enzyme structure into the solvent. A similar result was found in the complex formed by the wild type enzyme with thio-$NADP^+$ and trimethoprim which is also exclusively in form II.

In conclusion it can be noted that NMR studies of complexes of dihydrofolate reductase formed with modified ligands or proteins can clearly provide useful information about interactions, conformations and dynamic processes in such complexes in solution.

Acknowledgements—I would like to acknowledge the collaboration of Berry Birdsall and Gordon Roberts in all these studies and of other colleagues in various aspects of the work (J. Andrews, S. J. Benkovic, J. Jiminez-Barbero, A. Jimenez, M. T. A. Cheung, I. Kompis, S. Hammond and G. Ostler).

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