# Structural Comparisons of Complexes of Methotrexate Analogues with *Lactobacillus casei* Dihydrofolate Reductase by Two-Dimensional $^1$H NMR at 500 MHz

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ABSTRACT: We have used two-dimensional (2D) NMR methods to examine complexes of *Lactobacillus casei* dihydrofolate reductase and methotrexate (MTX) analogues having structural modifications of the benzoyl ring [the 3′,5′-difluoro and 3′,5′-dichloro analogues (II and III)] and also the glutamic acid moiety [the $\alpha$- and $\gamma$-monoamides (IV and V)]. Assignments of the $^1$H signals in the spectra of the various complexes were made by comparison of their 2D spectra with those of complexes containing methotrexate where we have previously assigned resonances from 32 of the 162 amino acid residues. In the complexes formed with the dihalomethotrexate analogues, the glutamic acid and pteridine ring moieties were shown to bind to the enzyme in a manner similar to that found in the methotrexate–enzyme complex. Perturbations in $^1$H chemical shifts of protons in Phe-49, Leu-54, and Leu-27 and the methotrexate H7 and NMe protons were observed in the different complexes and were accounted for by changes in orientation of the benzoyl ring in the various complexes (15° and 25° in the difluoro- and dichloromethotrexate complexes, respectively). Binding of oxidized or reduced coenzyme ($NADP^+$ or NADPH) to the binary complexes did not result in different shifts for Leu-27, Leu-54, or Leu-19 protons, and thus, the orientation of the benzoyl ring of the methotrexate analogues is not perturbed greatly by the presence of either oxidized or reduced coenzyme. In the complex with the $\gamma$-monoamide analogue, the $^1$H signals of assigned residues in the protein had almost identical shifts with the corresponding protons in the methotrexate–enzyme complex for all residues except His-28 and, to a lesser extent, Leu-27. This indicates that while the His-28 interaction with the MTX $\gamma$-$CO_2^-$ is no longer present in this complex with the $\gamma$-amide, there has not been a major change in the overall structure of the two complexes. This behavior contrasts with that seen for the $\alpha$-amide complex where $^1$H signals from protons in several amino acid residues are different compared with their values in the complex formed with methotrexate. The residues most affected are all near to the benzoyl ring of methotrexate: this indicates that the disruption of the $\alpha$-$CO_2^-$ interaction with Arg-57 is accompanied by a change in orientation of the benzoyl ring.

Methotrexate (I) is a well-known clinically useful cytotoxic agent that acts by inhibiting the enzyme dihydrofolate reductase. In the search for improved antifolate drug molecules, many methotrexate analogues have been synthesized and evaluated since the original synthesis of the parent compound [Seeger et al., 1949; also see review by Roth and Cheng (1982)]. More recently, there has been a considerable interest in detailed studies aimed at understanding the factors controlling the specificity of drug binding to the enzyme. One approach to investigating such specific interactions is to measure the change in binding energy that accompanies a structural modification of the drug aimed at removing or introducing a specific interaction. It is important that such binding studies be accompanied by structural studies to ascertain that the predicted changes in interaction have taken place and to investigate whether or not additional perturbations have been introduced. Previous studies on complexes of dihydrofolate reductase with trimethoprim analogues have used both X-ray (Kuyper et al., 1982) and NMR (Birdsall et al., 1984) methods to monitor the presence of predicted interactions in the complexes. While the X-ray method can give a very detailed description of the structures of the various complexes, NMR offers the advantage that it examines the complexes in solution, provides a convenient method for monitoring interactions, ionization effects, and dynamic processes, and, of course, does not require crystals. In the early NMR studies, considerations of the changes in chemical shift of the proton resonances of the ligand and of the histidine C2 proton resonances of the enzyme which accompanied complex formation allowed some details of the latter to be deduced. At the time of these studies the histidine signals were the only signals that had been assigned in the $^1$H spectrum of *Lactobacillus casei* dihydrofolate reductase (DHFR), and the value of this approach was thus limited. More recently, we have reported assignments of $^1$H signals to specific protons in 32 of the 162 amino acid residues in the enzyme (Hammond et al., 1986): these residues are widely distributed throughout the enzyme structure and can be used as a set of reporter groups to monitor changes in interactions, conformations, and ionization states within the protein–ligand complexes.

In this paper we report on studies of complexes of methotrexate analogues with *L. casei* DHFR and demonstrate how NMR data can be used to determine which parts of the complex show structural variations compared with the complex formed with the parent drug methotrexate. We have examined complexes formed with methotrexate analogues having structural modifications of the benzoyl ring [the 3′,5′-difluoro

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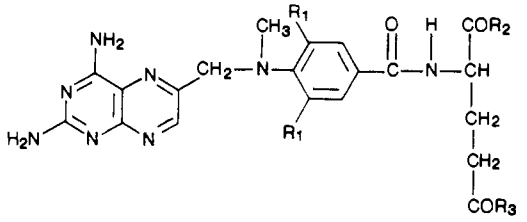
Chart I



| | | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| methotrexate (I) | MTX | H | OH | OH |
| 3′,5′-difluoromethotrexate (II) | $F_2$MTX | F | OH | OH |
| 3′,5′-dichloromethotrexate (III) | $Cl_2$MTX | Cl | OH | OH |
| methotrexate α-monoamide (IV) | MTX α-amide | H | $NH_2$ | OH |
| methotrexate γ-monoamide (V) | MTX γ-amide | H | OH | $NH_2$ |

and 3′,5′-dichloro analogues (II and III)] and also the glutamic acid moiety [the α- and γ-monoamides of methotrexate (IV and V)] as shown in Chart I.

The availability of crystal structure data on the ternary complex of the *L. casei* enzyme with MTX and NADPH (Matthews et al., 1978; Bolin et al., 1982) has proved to be of vital importance as an aid to assigning the protein $^1$H signals and in assisting the interpretation of the chemical shift perturbations seen in related complexes. From the crystal structure, the amino acid residues close to the methotrexate can be identified, and these are indicated in the partial structure shown in Figure 1. We have previously assigned $^1$H signals to specific protons in almost all of the residues shown in Figure 1 (Hammond et al., 1985). The shieldings of several of the protons in the amino acids shown in Figure 1 are directly influenced by ring current contributions from the pteridine and benzoyl rings of methotrexate. It is expected that any small relative movements of the methotrexate aromatic rings and the amino acid residues will result in chemical shift changes of the protons in these protein residues.

If the chemical shifts of any of these residues are the same in the complex formed with a methotrexate analogue as in the complex with methotrexate itself, then this provides strong evidence that the region of the complex structure containing these protons has not changed between the two ligands. Similar arguments can be applied to changes in chemical shifts observed for ligand protons that may have shielding contributions from ring currents of neighboring aromatic residues in the protein and also from aromatic rings within the ligand itself. Thus, for example, the H7 proton of methotrexate is expected to have ring current shift contributions from Phe-30 (−0.248 ppm), from Phe-49 (0.045 ppm), and from the benzoyl ring of methotrexate (−0.365 ppm).

Another way of characterizing the environment of a ligand proton is by measuring nuclear Overhauser enhancement (NOE) effects to its neighboring nuclei in the protein. Such methods can readily provide a semiquantitative comparison of conformations of related complexes although a precise definition of any differences is more difficult.

A further probe of structural changes is the measurement of the p$K$ values of ionizable groups suspected to be involved in the ligand–protein interaction. For example, the p$K$ of the imidazole ring of His-28, which can be conveniently monitored from the pH dependence of the appropriate imidazole C2 proton signal, can indicate whether or not the γ-carboxylate of the glutamic acid moiety of methotrexate analogues is interacting in the same way with His-28 in the different complexes (Antonjuk et al., 1984b; Birdsall et al., 1984).

## Experimental Procedures

*Materials and Methods.* Methotrexate was obtained from Lederle and from Nutritional Biochemical Corp. The 3′,5′-dihalomethotrexates, prepared by the method of Tomcufcik and Seeger (1961), were gifts from Dr. D. G. Johns and used without further purification. The methotrexate α-monoamide and methotrexate γ-monoamide were synthesized as described previously (Antonjuk et al., 1984a). $NADP^+$ and NADPH were obtained from Sigma Chemical Co. and used without further purification.

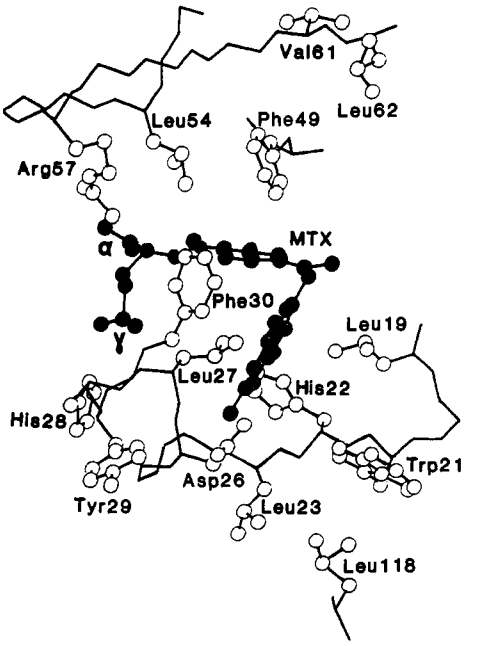



FIGURE 1: Part of the crystal structure of the MTX–NADPH–DHFR (*L. casei*) complex (Bolin et al., 1982) showing residues around the MTX binding site.

Dihydrofolate reductase was purified from *Lactobacillus casei* MTX/R as described previously (Dann et al., 1976) but with the addition of a Sephadex G-25 column as the final step to change the buffer concentrations for NMR work. We have checked the absence of NADPH by comparing the results of enzyme fluorescence quenching titrations using MTX and NADPH: the fact that the same molar amounts of each are required to quench the fluorescence is an indication that there is no NADPH impurity in the original preparation. We have also checked the absence of NADPH by adding folate to the enzyme and showing no change in its UV spectrum over 24 h and the absence of $NADP^+$ by monitoring the essential absence of the dismutase reaction when $FH_2$ is added to the enzyme. The absence of substantial amounts of $NADP^+$ or NADPH impurities has also been confirmed by the NMR $^1$H spectra (1D and 2D) of the enzyme–MTX complex (Hammond et al., 1986) where we do not detect any of the signals characteristic of the enzyme–MTX–$NADP^+$ and enzyme–MTX–NADPH complexes. All these complexes have been characterized separately (Hammond et al., 1986) and are in slow exchange on the chemical shift time scale: thus, the presence of mixtures of these complexes would lead to a superimposition of the component spectra each having nonintegral signals, and we see no evidence of this in our spectra. The purified enzyme was freeze-dried twice from $^2H_2O$ solution to remove exchangeable protons and then redissolved to give an approximately 1 mM enzyme solution in $^2H_2O$ containing 50 mM potassium phosphate/500 mM KCl/1 mM ethylenediaminetetraacetic acid (EDTA) pH* 6.5 (meter reading uncorrected for the isotope effect of the glass electrode). More concentrated solutions of the enzyme complexes (4 mM) were prepared by dialyzing the enzyme in the presence of the appropriate ligand at low salt concentration before

Table I: $^1H$ Chemical Shift Changes (ppm) Measured from the Corresponding Signals in MTX Binary and MTX·$NADP^+$ and MTX·NADPH Ternary Complexes[a]

| residue | | $F_2$MTX | $NADP^+$·$F_2$MTX | NADPH·$F_2$MTX | $Cl_2$MTX | $NADP^+$·$Cl_2$MTX | NADPH·$Cl_2$MTX | calcd ring current shifts: pteridine | calcd ring current shifts: PAB | gradient factor |
|---|---|---|---|---|---|---|---|---|---|---|
| Leu-19 | $C_{\delta_1}H_3$ | 0.04 | 0.07 | 0.06 | 0.07 | 0.07 | 0.04 | 0.22 | −0.06 | 0.04 |
| | $C_{\delta_2}H_3$ | 0.05 | 0.10 | 0.08 | 0.03 | 0.03 | 0 | −0.36 | | 0.10 |
| | $C_\gamma H$ | 0.01 | −0.03 | −0.02 | −0.06 | −0.06 | −0.15 | 0.10 | 0.07 | 0.03 |
| Leu-27 | $C_{\delta_1}H_3$ | 0.12 | 0.11 | 0.07 | 0.19 | 0.19 | 0.18 | 0.04 | −0.16 | 0.02 |
| | $C_{\delta_2}H_3$ | 0.21 | 0.23 | 0.19 | 0.32 | 0.34 | 0.28 | 0.19 | −0.87 | 0.13 |
| | $C_\gamma H$ | 0.13 | 0.12 | 0.08 | 0.17 | 0.17 | 0.16 | 0.12 | −0.22 | 0.02 |
| Trp-21[b] | $C_{\epsilon_3}H$ | −0.03 | 0 | −0.02 | −0.03 | 0.01 | | | | 0.23 |
| | $C_{\zeta_3}H$ | −0.01 | 0.01 | 0 | 0 | 0.04 | | −0.07 | | 0.02 |
| Phe-49 | $C_\delta H$ | 0.20 | 0.21 | | | | | 0.04 | −0.56 | 0.09 |
| | $C_\epsilon H$ | 0.26 | 0.17 | | | | | 0.08 | −0.04 | 0.05 |
| Leu-54 | $C_{\delta_1}H_3$ | 0.08 | 0.09 | 0.13 | 0.25 | | | −0.11 | −0.03 | 0.07 |
| | $C_{\delta_2}H_3$ | 0.08 | 0.06 | 0.06 | 0.13 | | | | −0.06 | 0.01 |
| | $C_\gamma H$ | 0.14 | 0.17 | 0.16 | 0.37 | | | | −0.15 | 0.04 |
| Val-61 | $C_{\gamma_2}H_3$ | −0.01 | 0.01 | −0.01 | −0.03 | −0.07 | −0.03 | | | 0.02 |
| | $C_{\gamma_1}H_3$ | 0.02 | 0.02 | −0.01 | −0.02 | −0.05 | −0.03 | | | 0.13 |
| | $C_\beta H$ | 0.02 | −0.01 | −0.02 | −0.01 | −0.07 | −0.04 | | | |
| Leu-62 | $C_{\delta_1}H_3$ | 0.03 | −0.01 | 0.01 | 0.01 | −0.03 | 0 | | | 0.10 |
| | $C_{\delta_2}H_3$ | −0.01 | −0.01 | −0.01 | 0 | −0.04 | −0.01 | | | 0.06 |
| | $C_\gamma H$ | 0 | −0.05 | −0.01 | 0.02 | −0.08 | −0.03 | | | 0.02 |
| Ile-96 | $C_\delta H_3$ | 0 | −0.05 | 0 | 0.02 | −0.06 | 0 | | | 0.11 |
| | $\{C_\gamma H$ | 0.01 | −0.09 | −0.01 | 0.01 | −0.17 | −0.04 | | | $\{0.03$ |
| | $\{C_\gamma H\}$ | 0.08 | 0.04 | −0.01 | 0.01 | 0.02 | −0.03 | | | $\{0.07\}$ |

[a] The chemical shifts of assigned protons in Leu-23, Ile-96, Ile-102, Leu-113, Leu-118, Met-128, Val-110, Val-115, Phe-103, and Trp-133 were also measured but showed no appreciable changes (<0.01 ppm) in the various complexes. Observed shifts underlined are those regarded to have changed significantly (>0.05 ppm). Positive shifts are downfield shifts. [b] The assignments for $H_{\epsilon_3}$ and $H_{\zeta_3}$ of Trp-21 (2.27 and 2.80 ppm, respectively) have been made on the basis of NOE connections of $H_{\epsilon_3}$ (Trp-21) to $H_{\delta_1,\delta_2}$ and $H_{\epsilon_1,\epsilon_2}$ of Phe-122: these signals had previously been tentatively assigned to Phe-30 (Hammond et al., 1986).

freeze-drying and redissolving in $D_2O$ to give the same buffer composition as indicated above. The complexes were formed by adding up to 2 molar equiv of the ligands to the enzyme solutions. Dioxane (1 mM) was added as a $^1H$ NMR chemical shift reference; its resonance is 3.75 ppm downfield of 5,5-dimethyl-5-silapentane-2-sulfonate at 308 K.

*NMR Spectroscopy.* The $^1H$ NMR spectra were recorded at 500 MHz with a Bruker AM500 spectrometer. The COSY, NOESY, and RELAY two-dimensional NMR spectra were recorded following the procedures described previously (Wider et al., 1983; Marion & Wuthrich, 1983; Bolton, 1982; Eich et al., 1982; Hammond et al., 1986; Searle et al., 1986).

The assignments of the $^1H$ signals in the spectra of the various complexes were made by comparisons of their 2D spectra with those of the corresponding complexes containing methotrexate. For signals that have the same chemical shift in the different complexes, this procedure can provide assignments with a high degree of confidence, particularly when the signals form complicated patterns of cross-peaks in their COSY and NOESY spectra which are the same in both complexes. Assignment is more difficult for signals that have different shifts in the various complexes: we have assigned such signals to protons that give signals in the enzyme–methotrexate complex having the same pattern of COSY and NOESY cross-peaks and showing the smallest differences in chemical shifts. Some of the shifted signals have NOE effects to known signals, which provide clear-cut assignments. The process is simplified because many of the signals remain unshifted, leaving a smaller set of signals to assign.

The pH dependences of the histidine C2 proton resonances were determined from one-dimensional NMR spectra and analyzed as described previously (Wyeth et al., 1980; Gronenborn et al., 1981; Antonjuk et al., 1984b).

We have calculated the ring current contributions to the $^1H$ shifts of all the protons in the protein by using the Johnson and Bovey (1958) equation and adopting an approach similar to that described by Perkins (1982). In order to evaluate which protons are in regions where the ring current shift contributions are particularly sensitive to small spatial movements, we have calculated a "shift gradient factor" relating ring current shifts to changes in position for each of the protons. For each aromatic ring in the structure we calculated the change in the ring current shift contribution for every proton when the proton is displaced by ±0.2 Å in the $p$ and $z$ directions in a cylindrical coordinate system with its origin at the center of the ring (Johnson & Bovey, 1958) and then averaged the absolute value of these changes. For each proton, the gradient factors from all rings were summed to give the values in Table I. The calculated gradient factors are given for the assigned protons listed in Table I; the protons with the largest values will have chemical shifts that are the most susceptible to small spatial movements.

## Results and Discussion

*3′,5′-Dihalomethotrexate Complexes.* Methotrexate binds very tightly to dihydrofolate reductase, and only an upper limit for its $K_d$ value ($<10^{-11}$ M) can be determined. Fluorescence quenching experiments lead to values of $K_d$ of 1.7 nM and 4 nM for $F_2$MTX and $Cl_2$MTX, respectively, indicating that they are binding less tightly.

Figure 2 shows the aliphatic regions of the $^1H$ 500-MHz NMR spectra of the complexes of the enzyme with methotrexate and its dihalo analogues. The detailed assignments for several protein and ligand residues, obtained as described earlier, are reported in Tables I and II. A comparison of the data reveals that some of the signals show appreciable changes in chemical shift (≥0.05 ppm) while others remain constant in the different complexes. For the purpose of the present discussions, protons having signals that are perturbed by less than 0.05 ppm from the chemical shifts in the enzyme–MTX complex are considered not to be influenced by the change in structure.

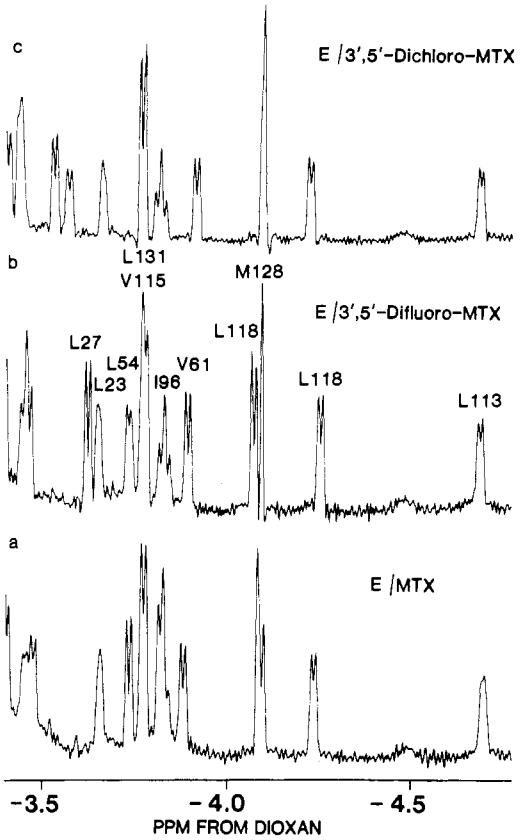



FIGURE 2: High-field region of the resolution-enhanced 500-MHz $^1H$ NMR spectra of complexes of dihydrofolate reductase: (a) E·MTX; (b) E·$F_2$MTX; (c) E·$Cl_2$MTX.

Table II: $^1H$ Chemical Shifts of Ligand Signals in Binary Complexes of Methotrexate and Its 3',5'-Dihalo Analogues with Dihydrofolate Reductase and Their Ternary Complexes with NADPH and $NADP^+$ [a]

| complex | chemical shifts (ppm from dioxane) | | |
|---|---|---|---|
| | MTX H7 | MTX NMe | Ade A8 |
| E·MTX | 4.14 | −0.30 | |
| E·MTX·$NADP^+$ | 4.09 | | 4.30 |
| E·MTX·NADPH | 4.19 | | 4.23 |
| E·$F_2$MTX | 4.42 | −0.45 | |
| E·$F_2$MTX·$NADP^+$ | 4.32 | | 4.28 |
| E·$F_2$MTX·NADPH | 4.46 | | 4.22 |
| E·$Cl_2$MTX | 4.27 | −0.57 | |
| E·$Cl_2$MTX·$NADP^+$ | | | 4.20 |
| E·$Cl_2$MTX·NADPH | 4.31 | | 4.21 |

[a] Positive shifts are downfield of the reference.

It is seen that the limited number of residues that show appreciable chemical shift changes are, with the exception of Ile-96, all within the methotrexate binding site (see Figure 1). Phe-103, which makes substantial ring current shielding contributions to the Ile-96 γ- and δ-protons, is on part of the protein backbone which is near to the coenzyme (Gly-98/99): differences in the halo substitution on methotrexate clearly cause the coenzyme to interact differently with this part of the backbone and result in the observed shift changes of Ile-96 protons.

The p$K$ values of the His-28 residue are identical within experimental error (7.8 ± 0.1) in all three complexes, indicating that the γ-$CO_2^-$ group of the glutamic acid moiety is interacting with the charged imidazole ring of His-28 in the same way in all cases. It has already been established that this interaction is responsible for increasing the p$K$ of His-28 by 1 unit from its value in enzyme alone (Antonjuk et al., 1984b). In this previous study we found that this interaction apparently only takes place if the interaction between Arg-57 and the α-$CO_2^-$ group of the glutamic acid moiety has also been properly made. It thus appears that the glutamic acid moiety is binding similarly in the complexes of the enzyme with methotrexate and its 3',5'-dihalo analogues.

The $^1H$ signals of the assigned protons in Leu-19 and Trp-21 have essentially the same shifts in the different complexes: these protons are fairly close to the pteridine ring and in some cases have contributions to their shielding from ring currents in the pteridine ring (see Table I). These results provide a strong indication that there has been little or no movement of the pteridine ring with respect to these residues. We thus conclude that the pteridine ring which makes a major contribution to the overall binding energy is binding similarly in all three complexes. One-dimensional NOE experiments confirm this, since irradiation of the H7 proton of methotrexate or its dihalo analogues causes, in all three cases, intensity changes in three resolved resonances assigned to $CH_3$ groups of Leu-19 and -27, which are in close proximity to H7 in the crystal structure of the methotrexate–NADPH–enzyme complex (Bolin et al., 1982).

Examination of Table I indicates that the amino acid residues that have shown the largest perturbations in their proton signals in the enzyme–dihalomethotrexate spectra are those that have large ring current shift contributions from the *p*-aminobenzoyl ring. Thus, signals from Phe-49, Leu-54, and Leu-27 all show substantially different chemical shifts in complexes with difluoro- or dichloromethotrexate compared with the corresponding values in the complex with methotrexate.

The simplest structural change that could produce such shift perturbations would be a change in the orientation of the benzoyl ring about its $N_{10}$–$C_4$ axis. Such a movement would also change the chemical shifts of the MTX H7 and NMe protons, and indeed, these are seen to be very different in the three complexes (see Table II). We have calculated the ring current shift contributions for the H7 and NMe protons and also for the relevant protons in Phe-49, Leu-19, Leu-27, and Leu-54 for different orientations of the benzoyl ring assuming all other residues remain in the same conformation, and these are given in Table III. It is not possible to find one orientation of the benzoyl ring which can accurately account for all the observed shift differences simultaneously. However, the qualitative trends in the data can be reproduced by changes in orientation of 15° and 25° in the $F_2$MTX and $Cl_2$MTX complexes, respectively. The poor fit of the data is not too surprising since we are unable to make any allowance for the anisotropic shielding contributions of the halogen substituents themselves and also it seems likely that there will be some change in conformation of the neighboring amino acid residues. In particular, such a change in orientation of the benzoyl ring would cause the side chain of Phe-49 to move (Clore et al., 1984), which would in turn influence the shielding of the Leu-54 methyl protons.

We have also examined the ternary complexes of the methotrexate analogues with the enzyme and NADPH (and also with $NADP^+$), and the $^1H$ chemical shifts of the assigned resonances in these complexes are included in Table I. Of the assigned aliphatic resonances those of Leu-27, Leu-54, and, to a lesser extent, Leu-19 are the only ones that show substantial differences in chemical shift in the complexes with the MTX analogues. The observed differences are similar to those seen in the binary complexes, which suggests that the orientation of the benzoyl ring is not greatly perturbed by the presence of coenzyme in either its oxidized or its reduced form.

Table III: Observed and Calculated $^1H$ Chemical Shift Changes (ppm) Measured from the Corresponding Signals in the MTX Binary and MTX·NADPH Ternary Complexes[a]

| residue | | observed shift differences (ppm) | | | | calculated shift differences (ppm)[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $F_2$MTX | $F_2$MTX·NADPH | $Cl_2$MTX | $Cl_2$MTX·NADPH | 10° | 15° | 20° | 25° |
| Leu-19 | $C_{\delta_1}H_3$ | 0.04 | 0.06 | 0.07 | 0.04 | 0.05 | 0.06 | 0.09 | 0.11 |
| | $C_{\delta_2}H_3$ | 0.05 | 0.08 | 0.03 | 0 | 0.02 | 0.03 | 0.04 | 0.05 |
| | $C_\gamma H$ | 0.01 | 0.02 | −0.06 | −0.15 | 0.01 | 0.02 | 0.02 | 0.03 |
| Leu-27 | $C_{\delta_1}H_3$ | 0.12 | 0.07 | 0.21 | 0.18 | 0.09 | 0.13 | 0.20 | 0.30 |
| | $C_{\delta_2}H_3$ | 0.21 | 0.19 | 0.32 | 0.28 | 0.10 | 0.16 | 0.20 | 0.23 |
| | $C_\gamma H$ | 0.13 | 0.08 | 0.17 | 0.16 | 0.03 | 0.05 | 0.07 | 0.10 |
| Leu-54 | $C_{\delta_1}H_3$ | 0.08 | 0.13 | 0.25 | | 0.12 | 0.17 | 0.22 | 0.26 |
| | $C_{\delta_2}H_3$ | 0.08 | 0.06 | 0.13 | | 0.04 | 0.06 | 0.09 | 0.11 |
| | $C_\gamma H$ | 0.14 | 0.16 | 0.37 | | 0.04 | 0.06 | 0.09 | 0.14 |
| Phe-49 | $C_\delta H$ | 0.20 | | | | 0.00 | 0.01 | 0.05 | 0.09 |
| | $C_\epsilon H$ | 0.26 | | | | 0.12 | 0.18 | 0.22 | 0.26 |
| MTX | H7 | 0.28 | | 0.13 | | 0.06 | 0.13 | 0.19 | 0.30 |
| | NMe | −0.15 | | −0.27 | | −0.02 | −0.04 | −0.08 | −0.11 |

[a] Positive shifts are downfield shifts. [b] The shifts were calculated from the ring current shift contributions from the methotrexate benzoyl ring for different orientation about the $N_{10}$–$C_4$ axis compared with that in the MTX–NADPH–enzyme complex (Bolin et al., 1982).

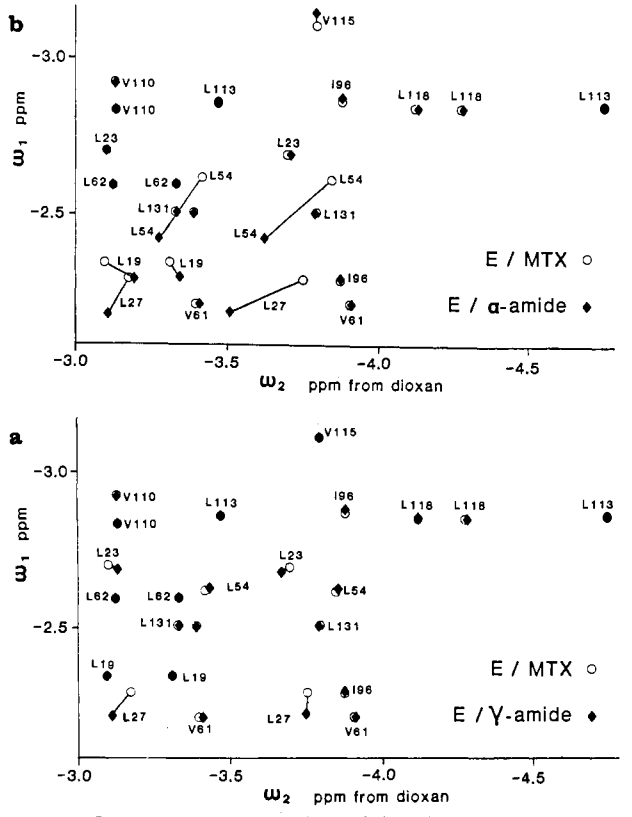



FIGURE 3: Schematic representations of the aliphatic regions of the 2D COSY $^1H$ contour plots for (a) E·MTX (○) and E·MTX γ-amide (♦) and (b) E·MTX (○) and E·MTX α-amide (♦).

The $C_\epsilon H$ resonance of Phe-49 in E·$F_2$MTX does show a change of −0.09 ppm on addition of $NADP^+$, but this is probably the result of a direct ring current shift contribution from the nicotinamide ring of the coenzyme.

The $^1H$ chemical shifts of the A8 protons of the adenine ring of bound $NADP^+$ and NADPH in the various complexes are reported in Table II. These are seen to be fairly constant in the NADPH complexes, indicating that the adenine ring binding site is not significantly affected by the changes in halo substituents on the methotrexate. For the ternary complex with $NADP^+$·$Cl_2$MTX there is a 0.1 ppm upfield shift of the A8 proton compared with the others: this complex is also unique in showing a shift difference for the resonances of Val-61. The larger change in orientation of the benzoyl ring in the $Cl_2$MTX complex may lead to a slight movement of the helix 42–49, which could affect the environment of these residues (Hammond et al., 1986).

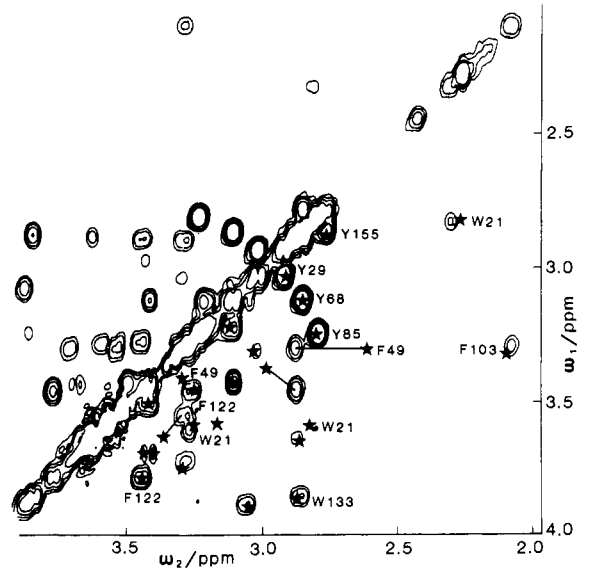



FIGURE 4: Aromatic region of the 2D COSY $^1H$ contour plot for the E·MTX α-amide: the assignments for the corresponding cross-peaks in the E·MTX complex are indicated (★) on the spectrum.

The chemical shift of the 7-proton of the bound methotrexate in its various analogoues was not influenced by the presence of NADPH although $NADP^+$ did cause small perturbations (~0.1 ppm), probably from a direct ring current shielding contribution from the nicotinamide ring (the nonaromatic reduced nicotinamide ring of NADPH would not show these effects).

*Methotrexate α- and γ-Monoamide Complexes.* Earlier measurements of $K_i$ values for these complexes indicated that the γ-amide analogue binds 9 times more weakly to the enzyme than does methotrexate while the α-amide binds 100 times more weakly (Antonjuk et al., 1984b). The $^1H$ chemical shifts of several assigned resonances in the spectra of the enzyme complexes formed with methotrexate and its monoamides are given in Figures 3 and 4. Figure 3 presents the data for the aliphatic regions of the spectra as schematic representations of the COSY contour plots. The signals from the γ-amide complex (Figure 3a) are seen to have almost identical chemical shifts with those in the methotrexate complex, thus indicating that there has been no major change in the overall structure of the two complexes (only Leu-27 shows a difference and this is a neighbor of His-28). This behavior contrasts with that observed for the methotrexate α-amide complex where the $^1H$ chemical shifts of several residues have changed appreciably compared with their values in the me-

thotrexate complex (see Figures 3b and 4): the residues most affected are Leu-27, Phe-49, Leu-54, and Leu-19, which are all near to the methotrexate benzoyl ring in the crystal structure of the enzyme–MTX–NADPH complex (Bolin et al., 1982). Figure 4 compares data from the aromatic region of the 2D COSY spectra of the enzyme complex with methotrexate with that of its complex with the $\alpha$-amide: it can be seen that the $\delta$-H chemical shift of Phe-49 has changed by 0.27 ppm in the $\alpha$-amide complex.

In an earlier study we investigated the glutamic acid moiety binding to the enzyme in complexes of the enzyme with these analogues (Antonjuk et al., 1984). We were able to monitor the interactions of the methotrexate glutamate $\gamma$-carboxylate group with His-28 by observing the pH dependence of the C2 proton of the imidazole ring of His-28 in the various complexes. We found that, as expected, modification of the $\gamma$-carboxylate to an amide abolishes its interaction with the imidazole of His-28. More surprisingly, the corresponding modification of the $\alpha$-carboxylate was found also to prevent the interaction of the $\gamma$-carboxylate with the imidazole of His-28. On the basis of these observations, we were able to make some approximate estimates of the contributions to the overall binding energy from the $\alpha$-carboxylate–Arg-57 and $\gamma$-carboxylate–His-28 interactions. In making these estimates, we assumed that there were no large conformational differences between the different complexes. The assumption that the pteridine ring was binding similarly in all three complexes has now been confirmed by one-dimensional NOE experiments involving irradiation of the methotrexate H7, which result in changes in intensity of aliphatic signals in the Leu-19/Leu-27 methyl region of the spectrum. The $^1$H chemical shifts reported here clearly indicate that the benzoyl ring has the same orientation in the methotrexate and $\gamma$-amide complexes but that its orientation is different in the complex with the $\alpha$-amide. The almost identical chemical shifts observed in the methotrexate and $\gamma$-amide complexes strongly confirm our original view that the disruption of the glutamate $\gamma$-$CO_2^-$–His-28 interaction involves only a local perturbation of the structure of the complex and does not appear to have any long-range conformational implications.

In our earlier study (Antonjuk et al., 1984b) we tentatively suggested that the disruption of the glutatmic $\alpha$-$CO_2^-$–Arg-57 interaction also causes a change in orientation of the benzoyl ring. This is now very clearly confirmed by the observations that the chemical shifts of protons in Leu-27, Phe-49, Leu-54, and Leu-19 are all perturbed when compared to their values in the enzyme–methotrexate complex: these are the protons in the residues most affected by ring current shielding contributions from the benzoyl ring.

In conclusion, we note that the availability of a large number of assigned NMR "reporter groups" from residues widely distributed throughout the enzyme provides the basis of a very sensitive fingerprint method for detecting differences in the mode of binding of a series of structural analogues to the receptor. The more precise definition of the conformational changes in the various complexes which is needed to account quantitatively for the differences in binding energy will require detailed quantitative NOE studies.

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