NMR Studies of Multiple Conformations in Complexes of *Lactobacillus casei* Dihydrofolate Reductase with Analogues of Pyrimethamine†

B. Birdsall,‡ S. J. B. Tendler,‡ J. R. P. Arnold,§ J. Feeney,*,‡ R. J. Griffin,⊥ M. D. Carr,‡ J. A. Thomas,§ G. C. K. Roberts,§ and M. F. G. Stevens⊥

Laboratory of Molecular Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom, Biochemistry Department, University of Leicester, Leicester LE1 7RH, United Kingdom, and Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, United Kingdom

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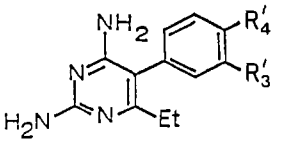
ABSTRACT: ^1H and ^{19}F NMR signals from bound ligands have been assigned in one- and two-dimensional NMR spectra of complexes of *Lactobacillus casei* dihydrofolate reductase with various pyrimethamine analogues (including pyrimethamine [**1**, 2,4-diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine], fluoropyrimethamine [**2**, 2,4-diamino-5-(4′-fluorophenyl)-6-ethylpyrimidine], fluoronitropyrimethamine [**3**, 2,4-diamino-5-(4′-fluoro-3′-nitrophenyl)-6-ethylpyrimidine], and methylbenzoprim [**4**, 2,4-diamino-5-[4′-(methylbenzylamino)-3′-nitrophenyl]-6-ethylpyrimidine]). The signals were identified mainly by correlating signals from bound and free ligands by using 2D exchange experiments. Analogues (such as **1** and **2**) with symmetrically substituted phenyl rings give rise to ^1H signals from four nonequivalent aromatic protons, clearly indicating the presence of hindered rotation about the pyrimidine–phenyl bond. Analogues containing asymmetrically substituted aromatic rings (such as **3** and **4**) exist as mixtures of two rotational isomers (an enantiomeric pair) because of this hindered rotation and the NMR spectra revealed that both isomers (forms A and B) bind to the enzyme with comparable, though unequal, binding energies. In this case two complete sets of bound proton signals were observed. The phenyl ring protons in each of the two forms experience essentially the same protein environment (same shielding) as that experienced by the corresponding protons in bound pyrimethamine: this confirms that forms A and B correspond to two rotational isomers resulting from ~180° rotation about the pyrimidine–phenyl bond, with the 2,4-diaminopyrimidine ring being bound similarly in both forms. The relative orientations of the two forms have been determined from NOE through-space connections between protons on the ligand and protein. Ternary complexes with $NADP^+$ were also examined: in the case of pyrimethamine and fluoronitropyrimethamine, conformations I and II, similar to those previously observed for enzyme complexes with trimethoprim and $NADP^+$, were detected in the ^{31}P spectra. The $NADP^+$ nicotinamide ring in form I binds within the protein with its glycosidic bond fixed in an anti conformation, while in form II the conformation of the pyrophosphate moiety is altered so that the nicotinamide ring extends away from the protein in a mixture of syn and anti conformations [Birdsall, B., Bevan, A. W., Pascual C., Roberts, G. C. K., Feeney, J., Gronenborn, A., & Clore, G. M. (1984) *Biochemistry 23*, 4733–4742]. For fluoronitropyrimethamine the conformational preference for binding is reversed in the binary and ternary complexes (the A/B ratio being 0.6/0.4 in the binary and 0.3/0.7 in the ternary complex). This is consistent with a model in which the conformational forms are correlated, with only forms IB and IIA being populated: the NO_2 substituent is oriented away from the nicotinamide ring binding site in form IB and toward the vacant site for nicotinamide ring binding in form IIA. Such a simple correlation cannot exist for the ternary complex of the enzyme with methylbenzoprim and $NADP^+$, which exists exclusively in form I but has both forms A and B significantly populated: when the preference for form I is high the correlation between forms A/B and I/II is less important.

Pyrimethamine **1** is representative of a series of 2,4-diaminopyrimidine inhibitors of dihydrofolate reductase, which are of continuing therapeutic interest as antimalarial (Roth & Cheng, 1982) and potential antitumor agents (Hull & Price, 1980; Bliss et al., 1987; Griffin et al., 1989). Because of their biphenyl-like structure, pyrimethamine analogues can give rise to rotational isomers resulting from hindered rotation about the pyrimidine–phenyl bond. Such restricted rotation is well-known in ortho-substituted biphenyls (Meyer & Meyer, 1963) and one would expect ortho-substituted phenylpyrimidines to behave similarly. Understanding the binding of these rotameric forms to DHFR[1] is important in under-



	R'_4	R'_3
1	Cl	H
2	F	H
3	F	NO_2
4	$N(CH_3)(CH_2Ph)$	NO_2

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‡National Institute for Medical Research.
§University of Leicester.
⊥Aston University.

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standing the structure–activity relationships of these ligands. To gain further insight into the phenomenon we have been using NMR spectroscopy to obtain structural information about complexes of *Lactobacillus casei* DHFR with pyrimethamine (**1**) and some related analogues **2–4** (Chart I) (Bliss et al., 1987; Griffin et al., 1989). Analogues containing asymmetrically substituted aromatic rings (such as **3** and **4**) can exist as mixtures of two rotational isomers (an enantiomeric pair) and one might expect some conformational selection of the rotamers on binding to DHFR. We have previously used ^{19}F NMR spectroscopy to show that fluoronitropyrimethamine (**3**) binds to DHFR in two different conformations and we suggested that these conformations correspond to the two rotameric forms. The populations of the two bound forms (A and B) were different, the A/B ratio being 0.6/0.4 in the binary state and 0.3/0.7 in the ternary complex with $NADP^+$ (Tendler et al., 1988). We have now used ^1H NMR spectroscopy to characterize the binary and ternary complexes of these compounds in more detail in order to determine unequivocally the structures of the different bound forms and to explain the change in preference that the enzyme has for the two rotamers in the binary and ternary complexes.

Materials and Methods

Pyrimethamine [**1**, 2,4-diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine] was obtained from Sigma and used without further purification. Fluoropyrimethamine [**2**, 2,4-diamino-5-(4′-fluorophenyl)-6-ethylpyrimidine], fluoronitropyrimethamine [**3**, 2,4-diamino-5-(4′-fluoro-3′-nitrophenyl)-6-ethylpyrimidine], and methylbenzoprim [**4**, 2,4-diamino-5-[4′-(methylbenzylamino)-3′-nitrophenyl]-6-ethylpyrimidine] were prepared and purified by methods described previously (Bliss et al., 1987; Griffin et al., 1989).

L. casei DHFR was obtained from *Escherichia coli* into which the *L. casei* gene had been cloned (Andrews et al., 1985). The protein was isolated and purified by using minor modifications of the method described previously (Dann et al., 1976). The enzyme complexes of the lipophilic compounds were prepared by adding excess ligand to the solution of the free enzyme; the suspensions were stirred for 1 h and undissolved ligand was removed by centrifugation. Final samples of the complexes contained 1–3 mM protein dissolved in D_2O in 500 mM KCl and 50 mM potassium phosphate at pH* 6.5 (pH* denotes a pH reading uncorrected for any deuterium isotope effect on the glass electrode). Some samples contained 0.3 mM dioxan as an internal chemical shift reference (the dioxan resonance is 3.75 ppm downfield of 5,5-dimethyl-5-silapentane-2-sulfonate at 308 K). ^1H NMR spectra were recorded at 308 K by using Bruker AM500 and AM400 spectrometers. The ^{19}F and ^{31}P spectra were obtained by using a Bruker AM400 spectrometer.

The ^1H COSY, DQF-COSY, NOESY (Ernst et al., 1987), and HOHAHA (Bax & Davies, 1985) experiments were all recorded with the carrier placed on the residual HDO signal, the latter being set at the center of the spectrum in both dimensions. Quadrature detection in F_1 was obtained by incrementation of the phase of the preparatory pulse by the TPPI method (Marion & Wuthrich, 1983; Williamson et al., 1984). A spectral width of 12.8 ppm was normally used in both dimensions. Usually 1024–4096 t_2 data points were recorded for each of 256–512 t_1 experiments, with suitable zero filling in t_2 and t_1 being carried out prior to Fourier transformation. Some of the COSY spectra were obtained in the absolute value mode using a $(90\text{–}t_1\text{–}90\text{–}t_2\text{–}d)_n$ pulse sequence with a 16-step phase cycle to select *N*-type peaks and to suppress quadrature images and axial peaks (Wider et al., 1984).

For COSY, HOHAHA, and NOESY spectra recorded in ^2H_2O the residual solvent peak was suppressed by preirradiation during the relaxation delay. The water peak was not irradiated during t_1 in order to avoid Bloch-Seigert shifts in F_1. The NOESY spectra were recorded with mixing times of 100 or 150 ms [each with a small random variation (±10 ms) in order to suppress zero-quantum coherence].

The equilibrium binding constants for the formation of binary complexes of pyrimethamine analogues with *L casei* DHFR were measured fluorometrically by measuring the quenching of the tryptophan fluorescence of the protein (pyrimethamine, $K_a = 5 \times 10^5\ M^{-1}$; fluoronitropyrimethamine, $K_a = 1 \times 10^6\ M^{-1}$; methylbenzoprim $K_a = 5 \times 10^6\ M^{-1}$). The binding constants for the ternary complexes were estimated by using etheno $NADP^+$, which has enhanced fluorescence when bound to the enzyme ligand complexes (Birdsall et al., 1980): these were within a factor of 2 of those for the binary complexes.

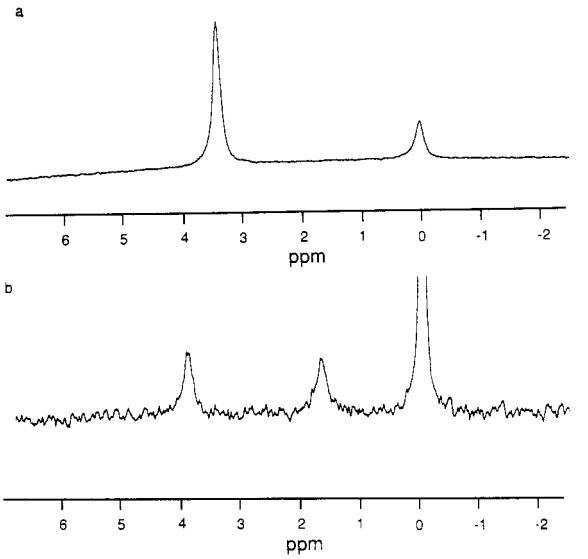


FIGURE 1: ^{19}F spectra of the complexes of *L. casei* DHFR with fluoropyrimethamine (a) and fluoronitropyrimethamine (b). Both spectra are referenced to the signal from excess free ligand present in the samples (376 MHz at 308 K and pH 6.5).

Results

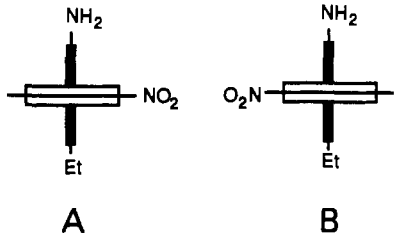
^1H Chemical Shifts of the Aromatic Protons in the Bound Ligands. All four pyrimethamine analogues **1–4** bind tightly to *L. casei* DHFR and exchange between bound and free ligands is expected to be slow, so that separate NMR spectra should be seen for the bound and free species. This is seen most clearly in the ^{19}F NMR spectra of DHFR complexes formed with fluorine-containing ligands. For example, the ^{19}F spectrum of the fluoropyrimethamine·DHFR complex, recorded in the presence of excess ligand, shows two separate signals corresponding to bound and free fluoropyrimethamine (Figure 1a). In the corresponding spectrum of the fluoronitropyrimethamine·DHFR complex two signals are seen for the bound ligand in addition to that for the free fluoronitropyrimethamine (Figure 1b). The two signals from the bound ligand were attributed to two different bound conformations and it was suggested that these corresponded to the rotational

[1] Abbreviations: 2D, two dimensional; COSY, two-dimensional correlation spectroscopy; DAP, 2,4-diaminopyrimidine; DHFR, dihydrofolate reductase; DQF–COSY, double quantum filtered correlation spectroscopy; enzymes, dihydrofolate reductase (EC 1.5.1.3.); HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; TPPI, time-proportional phase incrementation.

Table I: ^{1}H Chemical Shifts (ppm from Dioxane Reference) in Complexes of Pyrimethamine Analogues with *L. casei* DHFR

compound	chemical shifts (free) (ppm)				conformation	chemical shifts (bound) (ppm)				difference shifts (free − bound)			
	H2′	H3′	H5′	H6′		H2′	H3′	H5′	H6′	H2′	H3′	H5′	H6′
pyrimethamine	3.55	3.79	3.79	3.55		4.20	3.90	3.33	2.12	−0.65	−0.11	0.46	1.43
fluoropyrimethamine	3.54	3.61	3.61	3.54		4.15	3.63	3.05	2.05	−0.61	−0.02	0.56	1.49
fluoronitropyrimethamine	4.39		3.84	3.98	A	4.87		3.29	2.39	−0.48		0.55	1.59
					B	2.50		4.06	4.60	1.89		−0.22	−0.62
methylbenzoprim	4.07		3.58	3.56	A	4.52		2.67	1.88	−0.45		0.91	1.68
					B	2.42		3.55	4.18	1.65		0.03	−0.62
fluoronitropyrimethamine + $NADP^+$	4.39		3.84	3.98	A								
					B	2.30		4.07	4.74	2.09		−0.23	−0.76
methylbenzoprim + $NADP^+$	4.07		3.58	3.56	A			2.71	1.90			0.87	1.66
					B			3.84	4.50			−0.26	−0.94

Chart II



isomers A and B (Chart II), which do not interconvert because of hindered rotation about the pyrimidine–phenyl bond (Tendler et al., 1988). In this present ^{1}H study we have observed hindered rotation of this type for all the enzyme complexes of pyrimethamine analogues studied. Thus, in complexes where we have a symmetrically substituted phenyl ring, such as pyrimethamine and fluoropyrimethamine, signals from four nonequivalent aromatic protons were detected for the bound ligand, while for the complexes containing analogues with asymmetrically substituted phenyl rings, such as fluoronitropyrimethamine (**3**) and methylbenzoprim (**4**), two complete sets of bound proton signals were observed for the phenyl ring protons. A comparison of the chemical shifts for the phenyl ring protons in the various complexes can provide valuable information about the two conformations in the DHFR complexes.

Pyrimethamine·DHFR. The regions of the COSY and NOESY spectra of the pyrimethamine·DHFR complex containing resonances from aromatic protons are shown in Figures 2 and 3.

The signals from the four nonequivalent aromatic protons in bound pyrimethamine were identified in the 2D COSY spectrum of the complex by observing the intense cross-peaks connecting the coupled ortho protons H2′,H3′ and H5′,H6′. Corresponding cross-peaks were also observed in the 2D NOESY spectrum of the complex; this spectrum contained additional cross-peaks, arising from chemical exchange, connecting the aromatic proton signals from bound pyrimethamine with the corresponding signals in free pyrimethamine. Since the assignments of these protons in free pyrimethamine have been determined, the 2D exchange peaks from the NOESY spectrum provide unequivocal assignments for the four nonequivalent aromatic protons in bound pyrimethamine. The ^{1}H chemical shifts for the bound and free ligand are given in Table I.

Fluoropyrimethamine·DHFR. In the ^{1}H COSY spectrum of this complex it was also possible to identify signals from four nonequivalent aromatic protons in the bound ligand. In this case, the ^{1}H NOESY spectrum could not be used to connect signals from bound and free ligand by chemical exchange because the amount of free species present was too small due to the insoluble nature of the compound; the four

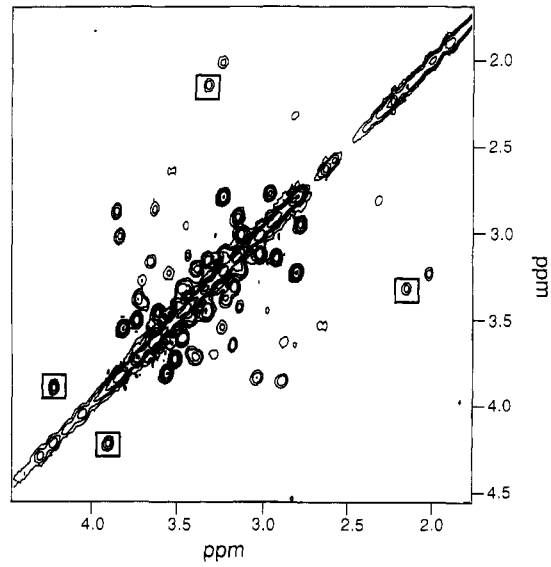


FIGURE 2: Aromatic region of the ^{1}H COSY spectrum of the pyrimethamine·DHFR complex (500 MHz at 308 K and pH 6.5).

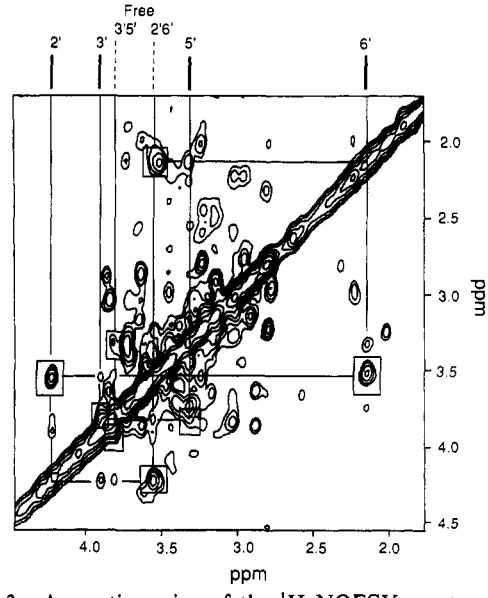


FIGURE 3: Aromatic region of the ^{1}H NOESY spectrum of the pyrimethamine·DHFR complex in the presence of free pyrimethamine. The 2D exchange cross-peaks connecting aromatic signals in free and bound species are labeled by boxes (500 MHz at 308 K and pH 6.5).

signals were assigned by comparison with the chemical shifts in the pyrimethamine·DHFR complex. The chemical shifts are included in Table I; when these are expressed as differences in chemical shift from the values in the free species they are seen to have very similar values to those for the pyrimethamine·DHFR complex (see Figure 5 below).

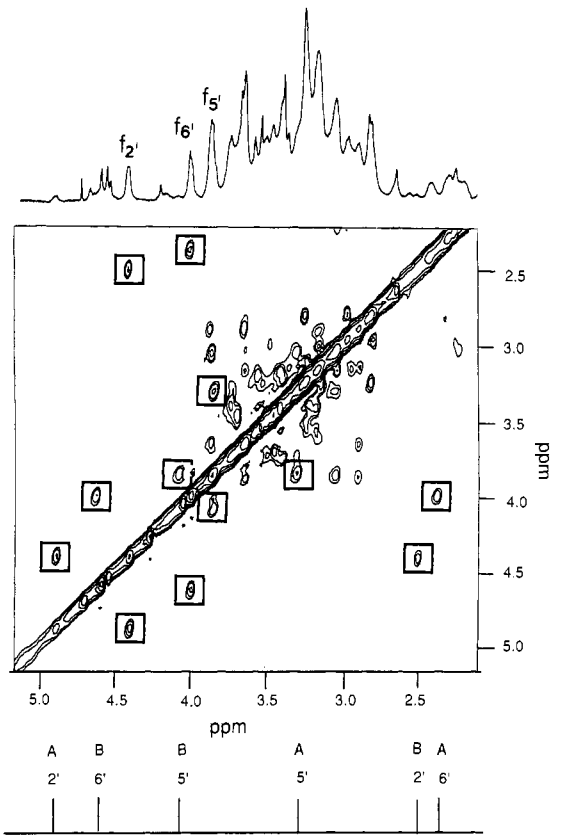


FIGURE 4: Aromatic region of the ^1H NOESY spectrum of the fluoronitropyrimethamine·DHFR complex in the presence of free fluoronitropyrimethamine. The 2D exchange cross-peaks connecting aromatic signals in the two bound species with the corresponding signals in the free ligand are labeled by boxes (500 MHz at 308 K and pH 6.5).

Fluoronitropyrimethamine·DHFR. Figure 4 shows the ^1H NOESY spectrum of this complex recorded in the presence of excess fluoronitropyrimethamine; six intense exchange cross-peaks connect the aromatic signals from the free ligand with two different sets of aromatic signals from the bound ligand. Since the signals in free fluoronitropyrimethamine can be assigned unambiguously, observation of these exchange peaks allows the assignment of the aromatic protons in the bound ligand. The two sets of bound signals indicate two conformations of the bound ligand, and that is consistent with the results of previous ^{19}F NMR studies (Tendler et al., 1988). Once these signals had been located in the 2D exchange experiment it was easy to identify them in the 1D ^1H spectrum of the complex (Figure 4). Some of the signals are sufficiently well resolved to allow their intensity to be estimated, and, thus, it was possible to correlate one set of bound signals (4.87 ppm, 3.29 ppm, 2.39 ppm) with the higher populated conformation (form A, 60%) as identified previously by ^{19}F NMR (Tendler et al., 1989).

We have also examined the ^1H COSY and NOESY spectra of the ternary complex fluoronitropyrimethamine·$NADP^+$·DHFR. In each case we could identify only one set of signals from bound fluoropyrimethamine at the sensitivity level of the experiment. The bound H5′ and H6′ signals in this set were also identified in the 1D ^1H spectrum and their chemical shifts (4.07 ppm and 4.74 ppm, see Table I) indicated that they were from form B. Their intensity corresponded to a fractional population of 0.7 (±0.1) for form B. These results are fully consistent with the earlier ^{19}F study on the ternary complex where the A/B ratio was measured as 0.3/0.7 (Tendler et al., 1988).

Methylbenzoprim·DHFR. Methylbenzoprim (**4**), like fluoronitropyrimethamine, has an asymmetrically substituted aromatic ring providing a mixture of two rotational isomers, both of which could potentially bind to the enzyme. Examination of the ^1H COSY and NOESY spectra for this complex (not shown) revealed that signals for two sets of three nonequivalent aromatic protons can in fact be identified. Cross-peaks are seen between the ortho proton signals in both the COSY and NOESY spectra. In addition, exchange cross-peaks are seen between the corresponding signals from bound and free species in the NOESY spectrum. The ternary complex of methylbenzoprim·$NADP^+$·DHFR has also been examined: the ^1H COSY spectrum reveals H5′/H6′ cross-peaks for two bound forms (A and B) and their relative intensities indicate that both forms are significantly populated.

Chemical Shifts of Protein ^1H Resonances. It has been possible to assign many ^1H resonances from the protein in complexes of DHFR with tightly bound ligands such as methotrexate and trimethoprim [see, for example, Birdsall et al. (in press) and Hammond et al. (1986)]. It has proved possible to transfer these assignments to complexes of DHFR with the pyrimethamine analogues **1**–**4** by noting the similarities in connectivity patterns in COSY, HOHAHA, and NOESY spectra of the various complexes. Table II lists the ^1H chemical shifts of the assigned protein resonances from aromatic residues in the various complexes. The chemical shifts are very similar in the different pyrimethamine complexes, indicating that these complexes have very similar conformations. When the shifts are compared with those from the 2,4-diaminopyrimidine·DHFR complex, some differences are observed for resonances from Phe 30 and Phe 49, which are expected to be close to the phenyl ring binding site.

In the 2D NOESY spectrum of concentrated samples (data not shown) it was possible to detect cross-peaks connecting H6′ of bound pyrimethamine (form A) with the H4 and H3,5 protons of Phe 30.

^1H and ^{31}P NMR Studies of $NADP^+$ in Ternary Complexes. In the case of the ternary complexes, another form of multiple conformations must also be considered. We have shown previously that the ternary complex trimethoprim·$NADP^+$·DHFR and related complexes with trimethoprim analogues can exist in two different conformations (designated forms I and II) that differ in the binding of the nicotinamide ring (Gronenborn et al., 1981a,b; Birdsall et al., 1984).

The earlier studies characterizing the mixture of forms I and II showed that the best way of quantitating the equilibrium was to measure the intensities of the ^{31}P signals arising from the pyrophosphate phosphorus nuclei in $NADP^+$ in the two bound conformations (Gronenborn et al., 1981b; Birdsall et al., 1984). We have examined the ^{31}P spectra of the various ternary complexes involving $NADP^+$ and pyrimethamine analogues and in some complexes have found clear evidence for the two forms (I and II). For example, in the fluoronitropyrimethamine·$NADP^+$·DHFR complex there is 65% form I and 35% form II present, whereas the ternary complexes involving pyrimethamine (form I > 80%) and methylbenzoprim (form I > 90%) are largely present in one conformation (see Table III). In some cases it was possible to measure the ^1H chemical shifts for the bound $NADP^+$ from exchange cross-peaks between signals in bound and free species in the ^1H NOESY spectra (data not given). For the ternary complex with fluoronitropyrimethamine, the bound nicotinamide ^1H shifts measured in this way are characteristic of form II (N2, −0.13; N4, 0.11; N5, 0.02; N6, −0.02; A2, −0.90; A8, −0.62 ppm); presumably coenzyme bound in form I of the

Table II: ^{1}H Chemical Shifts (ppm from Dioxan Reference) for Protein Aromatic Ring Protons in Complexes of Pyrimethamine Analogues with *L. casei* DHFR

residue		2	1	3	2,4-diamino-pyrimidine[a]
F3	2,6	3.02	3.03	3.02	3.07
	3,5	3.28	3.29	3.29	3.30
	4	3.70	3.71	3.71	3.72
F30	2,6	3.17	3.15	2.87	3.07
	3,5	3.31	3.31	3.13	3.31
	4	3.44	3.44	3.18	3.48
F49	2,6	3.62	3.46	3.19	3.48
	3,5	3.37		3.38	3.48
	4	3.50	3.62	3.54	3.31
F83	2,6				3.64
	3,5				3.64
	4				
F103	2,6	2.02	2.02	2.03	2.03
	3,5	3.23	3.26	3.23	3.25
	4	3.55	3.56	3.54	3.60
F106	2,6	3.84	3.85	3.85	3.86
	3,5	3.03	3.03	3.04	3.03
	4	3.07	3.08	3.08	
F122	2,6	3.73	3.73	3.72	3.75
	3,5	3.39	3.38	3.38	3.43
	4	3.23	3.22	3.22	3.24
F136	2,6	3.23	3.22	3.22	3.23
	3,5	3.13	3.13	3.13	3.13
	4				
W5	4		3.46	3.46	3.45
	5		2.97	2.97	2.96
	6		2.88	2.88	2.87
	7		2.64	2.63	3.64
W21	4	2.31	2.33	2.31	2.38
	5	2.82	2.83	2.81	2.85
	6	3.65	3.66	3.62	3.69
	7	3.18	3.18	3.15	3.24
W133	4	1.92	1.91	1.91	1.85
	5		0.78		0.76
	6	2.89	2.89	2.89	2.89
	7	3.85	3.86	3.85	3.87
W158	4				
	5		3.62–3.69	3.69	3.69
	6		(3.43)	3.42	3.42
	7		3.50	3.49	3.52
Y29	2,6	3.14	3.14	3.15	3.09
	3,5	3.01	3.03	3.02	2.98
Y46	2,6		3.25		3.29
	3,5				
Y68	2,6	3.15	3.15	3.15	3.14
	3,5	2.91	2.92	2.92	2.91
Y85	2,6	3.23	3.23	3.23	3.24
	3,5	2.80	2.80	2.80	2.80
Y155	2,6	2.78	2.79	2.78	2.79
	3,5	2.95	2.96	2.95	2.89

[a] Birdsall et al., in press.

Table III: Percentage Populations of Forms I and II and the Pyrophosphate ^{31}P Chemical Shifts for the Ternary Complexes of DHFR with NADP$^+$ and Pyrimethamine Analogues

compound	populations[a]		^{31}P chemical shifts (ppm)[b]			
			form I		form II	
	form I	form II				
pyrimethamine	80	20	−16.1	−14.9	−14.9	−12.4
fluoronitro-pyrimethamine	65	35	−16.5	−14.8	−14.8	−12.6
methylbenzoprim	>90	<10	−16.3	−14.7		
trimethoprim	55	45	−16.9	−14.9	−14.9	−12.9

[a] Errors ±10%. [b] ^{31}P chemical shifts measured from 50 mM K_2HPO_4 solution (pH 8.0). Negative shifts are to high field. Errors on chemical shifts ±0.05 ppm. [c] From Birdsall et al. (1984).

complex is not exchanging rapidly enough with the free NADP$^+$ to be detected in the 2D exchange experiment. In the case of the ternary complex with methylbenzoprim, the bound nicotinamide ^{1}H shifts (N2, 0.80; N4, 0.32; N5, 0.01; N6, 0.42; A2, −0.89; A8, −0.47 ppm) are much closer to those for form I than form II of the trimethoprim·NADP$^+$·DHFR complex (Birdsall et al., 1984) but are not identical. This is not too surprising in view of the proximity of the phenyl ring of the ligand to the nicotinamide ring of NADP$^+$ in form I of the ternary complex.

Discussion

The Two Bound Conformations Are Rotational Isomers Arising from Hindered Rotation about the Pyrimidine–Phenyl Bond. The observation of four separate signals corresponding to four nonequivalent phenyl protons in the ^{1}H spectra of complexes of DHFR with pyrimethamine and fluoropyrimethamine indicates that the phenyl ring takes up a fixed orientation within the binding site: separate signals arise because the protons on opposite sides of the ring are in different environments. Baker et al., (1983) have obtained crystal structure data on the complex of pyrimethamine with *E. coli* DHFR and have shown that the 2,4-diaminopyrimidine ring of pyrimethamine occupies essentially the same binding site as that occupied by the corresponding ring in complexes of methotrexate and trimethoprim with the enzyme (Bolin et al., 1982; Baker et al., 1981) and that the phenyl ring of pyrimethamine is orientated at ~90° to the plane of the pyrimidyl ring. Based on this, a model of the complex of pyrimethamine with *L. casei* DHFR was constructed (see Figure 6) and from this it is clear the protons on one side of the phenyl ring are directed toward Phe 30. This has now been confirmed by observation of an NOE connection between an ortho proton on the phenyl ring of bound pyrimethamine and the H4 and H3,5 aromatic protons of Phe 30. If we assume that the side-chain conformation of Phe 30 is similar in the complexes formed with pyrimethamine and methotrexate, then ring current shift calculations indicate that one ortho-coupled pair of protons in pyrimethamine (H5′ and H6′ in form A) will have substantial upfield shift contributions from Phe 30 (2.1 ppm for H6′ and 0.27 ppm for H5′). This is semiquantitatively consistent with the large shifts observed for H6′ and the somewhat smaller values observed for H5′ in the complexes. These conformation-dependent chemical shift contributions to bound phenyl ring protons can best be assessed by considering the chemical shift differences between the protons in the bound and free species as presented in diagrammatic form in Figure 5. It is seen that for pyrimethamine in conformation A the H5′,H6′ pair of nuclei are those oriented toward Phe 30 and experiencing the upfield shift and this is consistent with the NOE data mentioned above. (Because of the symmetry of the phenyl ring in pyrimethamine and fluoropyrimethamine the bound conformation B is identical with conformation A although the labels on the two shielded protons have now become H2′,H3′. Had there been rapid interconversion between A and B only a single averaged signal would have been detected for H2′,H6′ and for H3′,H5′.) In the case of the complex with fluoronitropyrimethamine where we have an *asymmetrically* substituted phenyl ring, separate sets of signals are seen for the two forms A and B. A comparison of the bound chemical shift differences for these separate forms (Figure 5) with those for the pyrimethamine complex shows remarkable similarity. The same is true of the bound chemical shifts for the methylbenzoprim·DHFR complex. This indicates that the phenyl ring protons are experiencing essentially the same protein environment in each of the two forms as that experienced by the corresponding protons in pyrimethamine, which can only be true if forms A and B correspond to the two rotational isomers resulting from ~180° rotation about the pyrimidine–phenyl bond, the 2,4-diaminopyrimidine ring

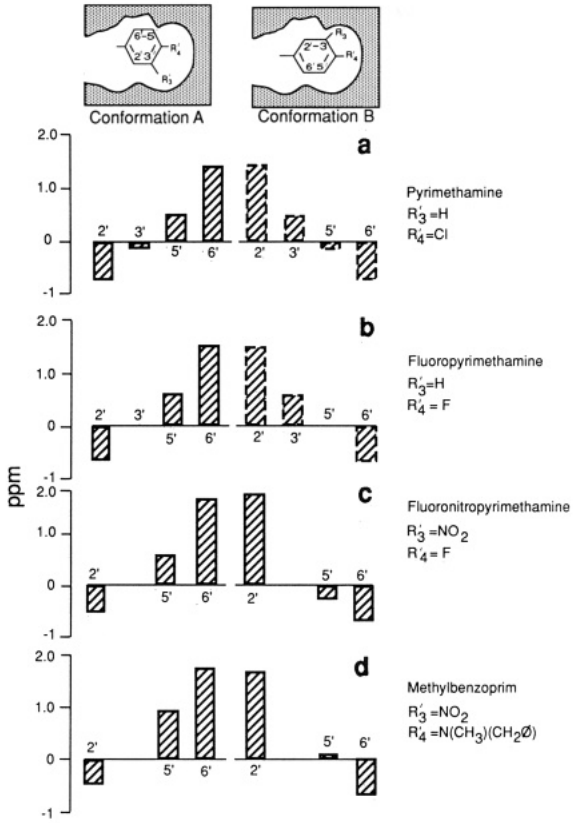


FIGURE 5: Schematic representation of the ^1H chemical shift differences between bound and free species for the aromatic protons in complexes of DHFR with pyrimethamine (a), fluoropyrimethamine (b), fluoronitropyrimethamine (c), and methylbenzoprim (d).

being bound similarly in both forms.

While the overall pattern of shielding contributions for the phenyl protons (see Table I and Figure 5) supports this general picture, a more detailed examination of the data indicates that there must be additional differences in the two conformations. The shielding contributions for nuclei in corresponding positions in the two forms (e.g., notably the meta protons) are closely similar but not identical; this can only be explained if there are differences in conformation in addition to the 180° rotation. The ^{19}F chemical shifts for the fluoronitropyrimethamine·DHFR complex confirm this. A simple 180° rotation about the pyrimidine–phenyl bond would leave the environment of the fluorine at the 4′-position unaltered, but in fact two separate ^{19}F signals are detected (see Figure 1b), again indicating additional conformational differences between the two forms. These could arise from several sources including (i) differences in the pyrimidine–phenyl torsion angle, i.e., rotation not exactly equal to 180° [this would result in differences in π-electron delocalization at the C-4′ position, a change of 30° giving $\sim$1 ppm shift contribution (Boden et al., 1964)], (ii) differences in the conformation of the NO_2 group, or (iii) differences in the protein conformation in the two forms. However, these additional conformational differences cannot be very large because of the similarity of the bound shifts seen for fluoropyrimethamine and fluoronitropyrimethamine complexes with DHFR. Furthermore, a detailed examination of the ^1H spectrum of the protein in each of these complexes did not reveal any protons giving rise to more than one signal: thus there are no extensive differences in protein conformation between forms A and B.

The conformational preference for binding form A in the fluoronitropyrimethamine·DHFR complex can be considered in terms of the model of the complex shown in Figure 6. In

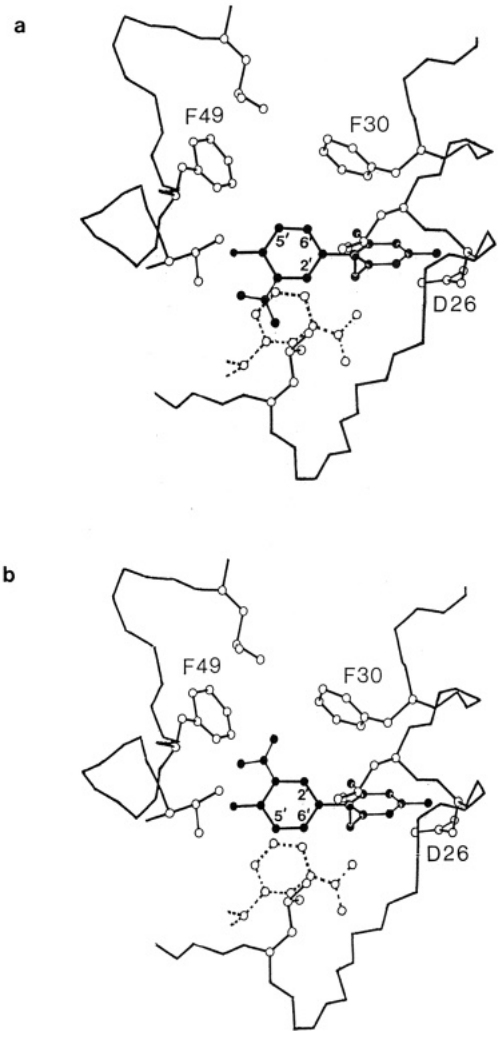


FIGURE 6: Model of the binding site in the fluoronitropyrimethamine·DHFR complex for (a) form A and (b) form B. The position of the $NADP^+$ nicotinamide ring binding site is also indicated as a dotted line structure. Modeling was carried out by using QUANTA (Polygen Inc.) on a Silicon Graphics IRIS 3130, incorporating crystallographic data from Matthews et al. (1978).

form A, the NO_2 substituent is directed toward the unoccupied coenzyme binding site in the binary complex and this could assist in favorable binding. Addition of $NADP^+$ to form the ternary complex reverses the preference for form A, which is consistent with unfavorable steric interactions between the bulky NO_2 group and the nicotinamide ring of bound $NADP^+$.

Correlation between Conformational States. The two conformations I and II in the fluoronitropyrimethamine·$NADP^+$·DHFR complex appear to be similar to those seen for the trimethoprim·$NADP^+$·DHFR complex, which have previously been characterized in some detail (Gronenborn et al., 1981a,b; Birdsall et al., 1984). The major difference between the two forms is in the behavior of the nicotinamide ring; in form I this is in a binding pocket within the protein with the glycosidic bond fixed in the anti conformation, while in form II the conformation of the pyrophosphate moiety is altered so that the nicotinamide ring extends away from the protein, in a mixture of syn and anti conformations. If the nicotinamide ring binding pocket in form I of the fluoronitropyrimethamine·$NADP^+$·DHFR complex is similar to that seen for NADPH (Bolin et al., 1982), then the nicotinamide ring will be close to the expected position of the phenyl ring of the pyrimethamine analogue. For this reason one must consider whether the populations of the rotational isomeric

conformations A and B of the asymmetrically substituted phenyl rings are correlated with the two conformational states of bound $NADP^+$ (forms I and II). Clearly the populations of forms I and II are influenced by the nature of the pyrimethamine analogues (see Table III) and furthermore populations of forms A and B are influenced by the presence of $NADP^+$. Thus one might expect some correlation between the different conformational states. If the forms are strongly correlated, then only two forms might be populated (for example IA and IIB or IB and IIA), while a weaker correlation would simply influence the population of all four forms (IA, IB, IIA, and IIB).

The ternary complex fluoronitropyrimethamine·$NADP^+$·DHFR is the only one for which the populations of forms A and B and I and II have all been estimated directly. In this case the results are the following: form A, 30; B, 70; I, 65; II, 35 ± 10%. In neither ^1H nor ^{31}P NMR spectra was there evidence for more than two conformations, and thus these results could be explained if the conformations are strongly correlated, with only forms IB and IIA being populated. This is consistent with a model where the NO_2 is oriented toward the vacant site for nicotinamide ring binding in form IIA and oriented away from the occupied nicotinamide ring binding in form IB (see Figure 6). However, one cannot exclude the alternative explanation that all four forms exist but that none of the monitored NMR resonances are affected by more than one of the conformational equilibria: thus signals that are affected by the A/B equilibrium are not affected by the I/II equilibrium and vice versa.

The results obtained for the ternary complex methylbenzoprim·$NADP^+$·DHFR are easier to interpret. In this case while the ^{31}P spectrum shows that the complex exists exclusively in form I, there is evidence in the ^1H COSY spectrum that forms A and B are both significantly populated. For this complex, then, there is clear evidence that forms A and B exist in the presence of form I: thus the occupancy of the nicotinamide ring binding site in this case does not preclude the binding of form A, where the NO_2 group is directed toward the nicotinamide ring binding site. It appears that when the preference for form I is high, the correlation between forms A/B and I/II is less important. Maybe the additional interactions formed by the benzyl ring of methylbenzoprim override any unfavorable steric interactions involving the NO_2 group and the nicotinamide ring. The ^1H chemical shifts of the bound H5′ and H6′ protons in form A are very similar for the binary and ternary complexes, indicating that the phenyl ring orientation remains unchanged. The chemical shift differences observed for H5′ and H6′ in form B for the binary and ternary complexes presumably result from ring curent contributions from the nicotinamide ring in the ternary complex.

Conclusion

Both rotational isomers of asymmetrically substituted pyrimethamine analogues have been observed to bind to dihydrofolate reductase with comparable, though not exactly equal, binding energies. This has implications for the design of modified pyrimethamine analogues as DHFR inhibitors. *L. casei* DHFR is not directly relevant to the likely therapeutic applications of pyrimethamine analogues, so that the present results cannot lead to specific recommendations for the design of improve therapeutic agents.

However, it is clear in general terms that changes in the substituents on the phenyl ring will have different effects on the binding of the two rotational isomers. The structure–activity relationship derived from, for example, K_i measurements will thus be composed of two independent structure–activity relationships, one for each isomer. A proper understanding of the binding of pyrimethamine analogues and the rational design of new analogues therefore requires that the equilibrium between the two rotational isomers be monitored.

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Partial ^{1}H NMR Assignments of the *Escherichia coli* Dihydrofolate Reductase Complex with Folate: Evidence for a Unique Conformation of Bound Folate†

Christopher J. Falzone,‡ Stephen J. Benkovic,*,‡,§ and Peter E. Wright*,§

Department of Chemistry, The Pennsylvania State University, 152 Davey Laboratory, University Park, Pennsylvania 16802, and Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT: Sequence-specific ^{1}H assignments have been made for over 25% of the amino acid side chains of *Escherichia coli* dihydrofolate reductase complexed with folate by using a variety of two-dimensional techniques. Proton resonances were assigned by using a combination of site-directed mutagenesis and a knowledge of the X-ray crystal structure. Unique sets of NOE connectivities present in hydrophobic pockets were matched with the X-ray structure and used to assign many of the residues. Other residues, particularly those near or in the active site, were assigned by site-directed mutagenesis. The ability to assign unambiguously the proton resonances of these catalytically important residues allowed for extensive networks of NOE connectivities to follow from these assignments. As a consequence of these assignments, the orientation of the pterin ring of folate could be determined, and its conformation is similar to that of the productive dihydrofolate complex. Under these experimental conditions, only one bound form of the pterin ring could be detected.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) is a monomeric protein consisting of 159 amino acids and has a molecular mass of 18 000 daltons. This enzyme catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F)[1] to 5,6,7,8-tetrahydrofolate (H_4F) and is responsible for maintaining intracellular levels of H_4F. Tetrahydrofolate and its derivatives are essential cofactors in one-carbon transfer reactions in a number of biosynthetic pathways. Thus, dihydrofolate reductase (DHFR) is an important target enzyme for antifolate drugs such as the antitumor agent methotrexate (MTX) and the antimicrobial agent trimethoprim.

The crystal structure for DHFR from *Escherichia coli* with the inhibitor MTX bound has been determined to a resolution of 1.7 Å (Bolin et al., 1982). It shows an abundance of secondary structure including four helices and an eight-stranded β-sheet. In comparing DHFR from *E. coli* to enzyme from other sources, it is observed that the secondary and tertiary structures are similar despite low primary sequence homology. For example, DHFR from *Lactobacillus casei* and *E. coli* share only a 28% primary sequence homology. However, some residues are strictly conserved or semiconserved (Glu for Asp substitution, for example), and when active site homology is compared, as many as 70% of the residues fall into these two categories for the NADPH site in the *L. casei–E. coli* comparison (Benkovic et al., 1988). Thus, there are certain key residues that occur in all species that have been optimized, at least under cellular conditions, for binding and catalysis. The role of these amino acids has been studied by combining site-directed mutagenesis with a detailed kinetic analysis in order to learn more about the relationship between structure and function.

The kinetic schemes for wild-type and several mutant DHFR's have been derived from pre-steady-state and steady-state kinetics (Fierke et al., 1987; Fierke & Benkovic, 1989; Murphy & Benkovic, 1989). Despite this detailed knowledge of the kinetic steps in the mechanism of DHFR and a knowledge of the stereochemistry of the reduction of dihydrofolate (Charlton et al., 1979), no other information is available about the structures of the substrates in the active sites of wild-type and mutant proteins and how these structures are related to the kinetic mechanism. We have begun to apply two-dimensional NMR methods to obtain information about the structure of the active site in *E. coli* DHFR and various

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‡ The Pennsylvania State University.
§ Research Institute of Scripps Clinic.

[1] Abbreviations: COSY, correlated spectroscopy; 2D, two dimensional; DHFR, dihydrofolate reductase; DTT, dithiothreitol; H_2F, 7,8-dihydrofolate; H_4F, 5,6,7,8-tetrahydrofolate; MTX, methotrexate; NOE, nuclear Overhauser effect; NOESY, two-dimensional proton nuclear Overhauser spectroscopy; 2Q, two-quantum spectroscopy; 2QF-COSY, double quantum filtered COSY; ROESY, rotating frame Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy.

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