^{13}C NMR Determination of the Tautomeric and Ionization States of Folate in Its Complexes with *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: ^{13}C NMR studies provide a convenient way of obtaining detailed information about tautomeric and ionization states in protein–ligand complexes provided that suitably ^{13}C-labeled molecules are available. In the present study, [4,6,8a-^{13}C]- and [2,4a,7,9-^{13}C]folic acid were synthesized and the ^{13}C NMR spectra of their complexes with *Lactobacillus casei* dihydrofolate reductase (DHFR) were assigned and analyzed as a function of pH. From these data it was possible to determine the tautomeric and ionization states of the bound folate and to obtain further evidence about the orientation of the pteridine ring in the complexes. In the ^{13}C spectra of the ternary complexes of the ^{13}C-labeled folic acids with DHFR and $NADP^+$, each labeled carbon gave rise to multiple signals, confirming our previous findings that there are three interconverting conformational forms of bound folate (forms I, IIa, and IIb) in the ternary complex (Birdsall *et al.*, 1989b). The ^{13}C spectra of the binary complexes of folate and DHFR also provide direct evidence for the presence of forms IIa and IIb and indirect evidence of some form I at low pH values (<5.0). 2D ^1H–^{13}C HMQC–NOESY experiments on ternary complexes formed using the [2,4a,7,9-^{13}C]folic acid were used to obtain intermolecular NOEs between the folate H7 proton and protons on the protein, and these provided further characterization of the orientations of the pteridine ring in the different bound forms of folate (form IIb with its pteridine ring in the catalytically active conformation and forms I and IIa with their pteridine rings turned over by 180°). The tautomeric and ionizable states of the pteridine rings in forms I, IIa, and IIb were investigated by comparing the ^{13}C chemical shifts of the pteridine ring carbons in the different forms with values obtained from "model" compounds with known tautomeric and ionization states and by considering ^1H chemical shifts of the enzyme complexes. Folate in form IIb, the catalytic active conformation of folate, exists in the keto form with N1 unprotonated whereas folate in form IIa and form I exists in enolic forms.

The enzyme dihydrofolate reductase (DHFR)[1] catalyzes the reduction of dihydrofolate (and folate with lower efficiency) to tetrahydrofolate using NADPH as a coenzyme. The enzyme plays a central role in maintaining the cellular pools of tetrahydrofolate derivatives, which are essential for the growth of the cell, and is of considerable pharmacological interest, being the target for "anti-folate" drugs such as trimethoprim and methotrexate (Blakely, 1985). Methotrexate is a close structural analogue of the substrate folate, and it was initially assumed that the inhibitor and substrate would bind to the enzyme in a very similar fashion. However, stereochemical studies (Hitchings & Roth, 1980; Fontecilla-Camps *et al.*, 1979; Charlton *et al.*, 1979, 1985) have shown that although the inhibitor and substrate occupy essentially the same binding site in their complexes with DHFR, the pteridine ring in the methotrexate complex is turned over by approximately 180° with respect to its orientation in the substrate complex. Subsequent NMR studies on complexes of folate with *Lactobacillus casei* dihydrofolate reductase indicated that in the DHFR–folate binary complex at least two different conformational states exist, and in the ternary complex DHFR–folate–$NADP^+$ three interconverting conformational states were found to be present (Birdsall *et al.*, 1981, 1987). The equilibrium between the three states was shown to be pH dependent, and the proportions of the states could be estimated by measuring the intensities of the ^{13}C resonances observed in the complex containing [*3-carboxamide*-^{13}C]$NADP^+$. At low pH, form I predominates, whereas at high pH, forms IIa and IIb predominante, with the ratio of these forms being relatively insensitive to changes in pH. The different conformational states were characterized using ^1H, ^{13}C, and ^{31}P NMR studies which indicated that forms I and IIa have conformations similar to each other (Birdsall *et al.*, 1982). Subsequent ^1H NOESY experiments on the ternary complex detected connections between the H7 proton of folate and assigned protein signals in the different conformational states: these confirmed that forms IIa and I had the pteridine ring in the "methotrexate-like" orientation with the ring turned over within the same binding site (Birdsall *et al.*, 1989b) whereas in form IIb the folate had its pteridine ring in the "active" conformation for catalysis ("folate-like"). The present work aims to extend these studies by determinig the tautomeric and ionization states of the pteridine ring system in the different bound forms of folate. This involves examining the ^{13}C spectra of complexes of *L. casei* dihydrofolate reductase formed with folates selectively enriched with ^{13}C at positions known to be

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[1] Abbreviations used: 2D, two-dimensional; 3D, three-dimensional; DHFR, dihydrofolate reductase; enzyme, dihydrofolate reductase (EC 1.5.1.3); HMQC, heteronuclear multiple quantum coherence spectroscopy; MTX, methotrexate; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy.

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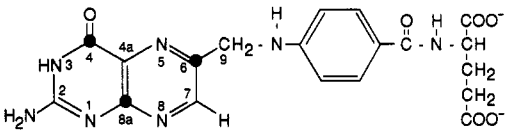
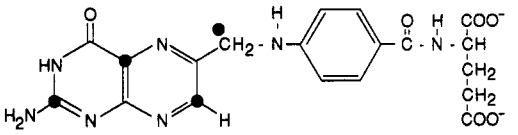
sensitive to the tautomeric and ionization states. Selinsky and co-workers (1990) have examined complexes of bovine DHFR formed with folic acids labeled with ^{13}C at C6 and ^{15}N at N5 and have shown that there is no protonation at N5 in these complexes. In the study reported here the two labeled folic acids **1** and **2** contain labeled ^{13}C atoms for all the carbons



(1) [4,6,8a-^{13}C] folate



(2) [2,4a,7,9-^{13}C] folate

on and adjacent to the pteridine ring: the shieldings of several of these are sensitive to the tautomeric state. The ^{13}C labeling at the proton-bearing C7 and C9 carbons of folate facilitates HMQC–NOESY experiments which allow detection of protein protons near the folate H7 and H9 and provide additional characterization of the different conformational states. Conference proceedings on some of the findings have appeared (Cheung *et al.*, 1992b).

MATERIALS AND METHODS

Dihydrofolate reductase was isolated from an *Escherichia coli* strain into which the *L. casei* dihydrofolate reductase gene had been cloned (Andrews *et al.*, 1985), and the enzyme was purified using methods described previously (Dann *et al.*, 1976). The syntheses of [2,4a,6-^{13}C]- and [4,7,8a,9-^{13}C]-methotrexate have been described previously (Cheung *et al.*, 1987).

The ^{13}C-labeled folic acids were prepared as follows. [4,6,-8a-^{13}C]-2,4-Diamino-6-methylpteridine and [2,4a,7,9-^{13}C]-2,4-diamino-6-methypteridine were first synthesized using procedures reported for similar ^{13}C-enriched analogues (Cheung & Gray, 1984) and then separately brominated at the 6-methyl as described earlier (Cheung *et al.*, 1987). The two individual bromination products were found by ^1H NMR analysis to consist of 65% monobromide and 35% unreacted material. Each bromination product from 50 mg (0.27 mmol) of the corresponding ^{13}C-enriched 2,4-diamino-6-methylpteridine was treated with a solution of 55 mg (0.17 mmol) of diethyl *N*-(*p*-aminobenzoyl)-L-glutamate and 0.01 mL of triethylamine in 1.0 mL of *N*,*N*-dimethylacetamide. The mixture was stirred at 55 °C for 5 h. A further 0.04 mL of triethylamine was added, and the solvents were removed under reduced pressure. The resultant residue was applied as a suspension in 2 mL of 1:19 methanol–chloroform to a short column of TLC-grade silica gel and chromatographed under suction to give 20 mg of aminopterin diethyl ester, ^{13}C-enriched at either the 4,6,8a- or the 2,4a,7,9-positions. The transformation of these multi-^{13}C-enriched aminopterin diethyl esters to the corresponding multi-^{13}C-enriched folic acids was carried out at 100 °C for 30 min using deoxygenated 1 M sodium hydroxide under an atmosphere of deoxygenated nitrogen and with protection from light. The reaction time was substantially shorter than that used for the conversion of aminopterin to folic acid (Seeger *et al.*, 1949; Selinsky *et al.*, 1990). Insoluble materials were removed by filtration while hot, and the filtrate after cooling was acidified by 1 M hydrochloric acid to pH 3.8. The solids formed were collected by filtration, washed with ice-cold water, and dried under vacuum. The yields of [4,6,8a-^{13}C]folic acid (**1**) and [2,4a,7,9-^{13}C]folic acid (**2**) were 60% and 48%, respectively.

Folic acid, $NADP^+$, and diethyl *N*-(*p*-aminobenzoyl)-L-glutamate were obtained from Sigma Chemical Co. 2-Amino-4-methoxy-6-methylpteridine and its 7-methyl and 7-methyl-9-nor analogues were prepared as described in the literature (Roth *et al.*, 1950). The pH measurements were carried out using a Radiometer Model PHM 83 pH meter equipped with a combination glass electrode. The reported pH values are the measured meter readings and are uncorrected for any deuterium isotope effects. Dioxane (0.1%) was used as an external standard (67.5 ppm from TMS at 298 K, 67.3 ppm at 281 K). The ^{13}C and ^1H NMR experiments were carried out on Varian UNITY 600, Bruker AM500, and AM400 NMR spectrometers. Most of the ^{13}C spectra were obtained under conditions of composite pulse proton decoupling using the WALTZ-16 sequence (Shaka & Keeler, 1987; Levitt *et al.*, 1983). The HMQC–NOESY experiment was carried out on the Bruker AM500 using the sequence proposed by Marion and co-workers (1989).

The samples of the DHFR complexes were prepared by redissolving (in ^2H_2O or 10% ^2H_2O/90% H_2O) freeze-dried enzyme containing 100–500 mM KCl and 10–50 mM potassium phosphate (final pH 6.5) and adding aliquots of concentrated solutions of labeled ligands. Some samples were further dialyzed in order to remove unbound ligand (for example, such samples were used for Figures 1–3 and 5). The DHFR–folate complexes were prepared with 100 mM KCl and 10–50 mM potassium phosphate, and the NMR experiments on binary complexes were performed at 281 K while the ternary complexes were studied at temperatures between 281 and 318 K. The DHFR–[4,6,8a-^{13}C]folate–$NADP^+$ sample used for the ^{13}C transfer of saturation experiment contained 165 mM KCl and 80 mM potassium phosphate at pH 6.0 and was examined at 318 K on the Varian UNITY 600-MHz spectrometer. The methotrexate complexes were made with 500 mM KCl and 50 mM potassium phosphate and examined at 298 and 308 K. The concentrations of the enzyme complexes examined were in the range 0.5–1.0 mM, and 1.8-mL samples were used for experiments run on the Bruker AM 400 spectrometer while 0.5-mL samples were used for those on the other instruments.

RESULTS

The ^{13}C chemical shifts of the ^{13}C-labeled folic acid and methotrexate were first measured as a function of pH in the absence of enzyme, and these results are included in Table I. Data were also obtained for 2-amino-4-methoxy-6-methylpteridine examined as a model compound for estimating the ^{13}C shifts for the enolic forms of a folate pteridine ring.

^{13}C Spectra of the Binary and Ternary Complexes Formed with L. casei DHFR

The ^{13}C NMR spectra of all the complexes formed with the labeled folates **1** and **2** show multiple signals for each labeled carbon in the bound folate, thus confirming the presence of the multiconformational states seen in our earlier studies (Birdsall *et al.*, 1981, 1982, 1987, 1989b). The pH dependence of the three conformational states in the ternary complex has already been well-characterized, and this information can be

Table I: ^{13}C Chemical Shifts of DHFR Substrates and Substrate Analogues Free and Complexed with *L. casei* DHFR

	C2	C4	C4a	C6	C7	C8a	C9
Bound Folate[a]							
DHFR–folate binary complex							
form IIa[b]	158.2	169.9	128–132	150.8	145.4	147.9	46.3[c]
form IIb	154.7	164.2	128.1	147.9	150.3	158.0	46.7[c]
DHFR–folate–$NADP^+$ ternary complex							
form I	159.8	171.2	123.9	152.1	150.5	151.3	45.5
form IIa	157.9	168.8	128–132	148.8	143.5	147.4	45.3
form IIb	154.9	163.3	129.5	145.2	150.6	158.9	46.1
Free Folate							
pH 10[d]	165.1	174.2	129.2	148.9	148.5	156.7	47.2
pH 5.5[d]	155.6	165.8	128.5	151.3	150.1	154.8	47.0
acidic solution[e]	152.1	158.7	127.8	152.7	147.8	147.4	45.8
Free Methotrexate							
pH 6.5[d]	162.6	164.1	123.4	149.9	150.1	153.5	56.0
pH 2.1[d]	156.7	164.1	123.2	152.8	150.2	145.7	56.0
Bound Methotrexate[f]							
DHFR–MTX binary complex	157.7	164.6	124.0	151.7	148.6	145.9	56.4
DHFR–MTX–$NADP^+$ ternary complex	157.9	164.6	123.3	151.7	149.6[g]	146.9	55.1
Free 2-Amino-4-methoxy-6-methylpteridine[h]							
neutral solution	161.0	168.8	121.6	148.1	151.4	155.5	
acidic solution	156.2	168.5	122.3	152.3	151.3	146.9	
Models							
keto model[i] (N1 unprotonated) **3**	155.6	165.8	128.5			154.8	
keto model[j] (N1 protonated) **4**	152.1	158.7	127.8			147.4	
enol model[k] (N1 protonated) **5**	156.6	167.1	124.0			147.3	
enol model[k] (N1 unprotonated) **6**	161.4	167.4	123.3			155.9	
enol model[l] (N1 protonated) **5**							
binary	156.5	167.7	126.4			147.3	
ternary	156.7	167.7	125.7			148.3	
enol model[l,m] (N1 unprotonated) **6**							
binary	161.3	168.0	125.7			155.9	
ternary	161.5	168.0	125.0			156.9	
enolate model[l] (N1 protonated) **7**							
binary	154.2	172.1	132.9			144.2	
ternary	154.4	172.1	132.2			145.2	
enolate model[l,m] (N1 unprotonated) **8**							
binary	159.0	172.4	132.2			152.8	
ternary	159.2	172.4	131.5			153.8	

[a] Measured at 281 K for about 0.5 mM solutions of the DHFR–folate (^{13}C-enriched) complex in 10–50 mM aqueous potassium phosphate and 100 mM potassium chloride containing 10% D_2O, and referenced to external dioxane at 281 K (67.3 ppm). [b] Data for form IIa refer to the spectrum acquired at pH 7.0. For shift changes at lower pH see text. [c] Assignments for C9 signals of forms IIa and IIb may be reversed. [d] Measured at 297 K for about 0.5 mM solutions of ^{13}C-enriched species in 10–50 mM aqueous potassium phosphate and 15–100 mM aqueous potassium chloride containing 10% D_2O, and referenced to 0.1% external dioxane at 297 K (67.5 ppm). Distinction between the signals of C6 and C8a in the spectra of [4,6,8a-^{13}C]folate at pH 10 and 5.5 (literature pK_a of folate ca. 2.4 and ca. 8.4) (Poe, 1973; Selinsky *et al.*, 1990) is by comparison with the literature data (Selinsky *et al.*, 1990) on [6-^{13}C]folate: pH 10, 148.0 ppm; pH 6, 150.0 ppm (shift change upon ionization at N_3/O_4 –2.0 ppm). [e] Measured at 295 K for a 0.1 M solution of unlabeled species in 4:1 v/v CH_3SOCH_3–$CDCl_3$ with or without 0.5 M CF_3COOH and referenced to CH_3SOCH_3 (40.4 ppm). Protonation of an organic solution by CF_3COOH was necessary due to problems in pH titrations at low pH [Selinsky *et al.*, 1990; Poe, 1973]. Signal assignments are as follows. In the absence of proton decoupling, C7 and C8a give rise to doublets with J_{C7H7} = 191 Hz and J_{C8aH7} = 12 Hz. The signal of C6 is broad due to couplings to H7 and H9, these couplings being 10 and 7 Hz, respectively, for the model compound [6-^{13}C]-2,4-diamino-6-methylpteridine (Cheung & Gray, 1984; Cheung *et al.*, 1987). The signal of C2 was assigned by a preliminary pH titration using [2,4a,7,9-^{13}C]folate. [f] Measured at 298 K for 0.5–1.0 mM solutions of the enzyme complex in 500 mM KCl and 50 mM potassium phosphate, pH 6.5, and referenced to external dioxane (67.5 ppm). [g] This value was incorrectly reported as 146.6 ppm in Cheung *et al.* (1992a). [h] Measured at 295 K for a 0.1 M solution of unlabeled species in 4:1 v/v CH_3SOCH_3–$CDCl_3$ with or without 0.5 M CF_3COOH and referenced to CH_3SOCH_3 (40.4 ppm). Signals were assigned by comparison with those of its 7-methyl and 7-methyl-9-nor analogues, and by noting couplings of C6 and C8a to H7. [i] Estimated from folic acid data at pH 5.5. [j] Estimated from folic acid data at low pH. [k] Estimated from data for 2-amino-4-methoxy-6-methylpteridine with N1 protonated and unprotonated and allowing for substituent effects OMe → OH (see text). [l] Estimated from data for methotrexate with N1 protonated bound to DHFR (in binary and ternary complexes) and allowing for substituent effects NH_2 → OH (enol) or NH_2 → O^- (enolate) (see text). [m] Effects of N1 deprotonation estimated from data for 2-amino-4-methoxy-6-methylpteridine obtained in neutral and acid solutions.

used to assign the multiple signals for each carbon to form I, IIa, or IIb simply by monitoring the signal intensities as a function of pH for the ternary complexes. The presence of no more than three labeled quaternary carbons in each folate usually makes the assignment of each signal to its carbon position relatively straightforward. However, in some cases where an ambiguity exists, this could be resolved by using transfer of saturation experiments to connect assigned signals in free folate with their correspnding signals in bound folate. This was particularly useful for assigning bound carbons C6 and C8a which have similar chemical shifts. In samples containing excess folate, separate signals were always detected for bound and free species, indicating the presence of slow exchange between these species.

L. casei DHFR–[4,6,8a-^{13}C]Folate–$NADP^+$ Ternary Complex. The ^{13}C spectra which are easiest to assign are those of the ternary complex of DHFR formed with [4,6,8a-^{13}C]-folic acid at different pH values (see Figure 1). Each of the three labeled pteridine carbons gives rise to a pair of ^{13}C signals at pH 6.8. These correspond to forms IIa and IIb as discussed previously, and the larger component of each pair can be assigned to the more abundant form IIb. As the pH is lowered, three additional signals corresponding to form I appear in the spectrum and grow in intensity at the expense of the other

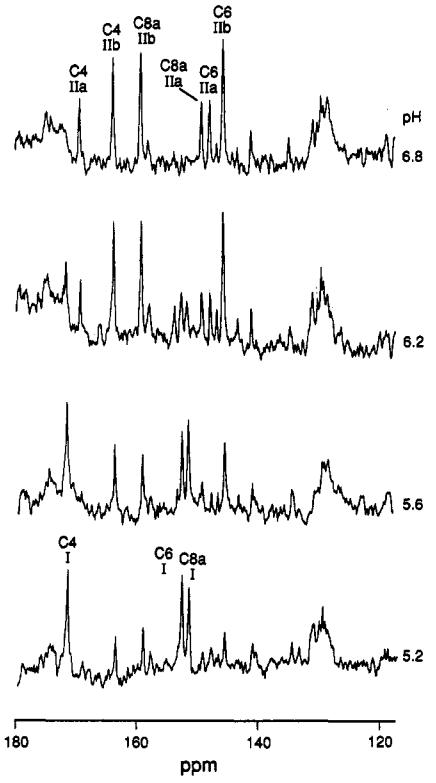


FIGURE 1: Low-field region of the 100.6-MHz ^{13}C NMR spectra at 281 K of the ternary complex of *L. casei* DHFR and NADP$^+$ and [4,6,8a-^{13}C]folic acid at different pH values. Signals arising from the naturally occurring ^{13}C in the protein are also observed (~130, 157, and ~175 ppm).

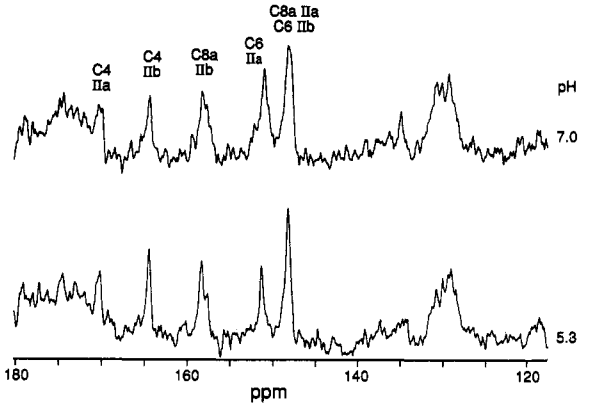


FIGURE 2: Low-field region of the 100.6-MHz ^{13}C NMR spectra at 281 K of the binary complex of *L. casei* DHFR with [4,6,8a-^{13}C]folic acid at pH 7.0 and 5.3.

signals. The signals can thus be assigned to forms IIa, IIb, and I by comparing their pH behavior with that predicted for these forms from results of previous studies.

L. casei DHFR–[4,6,8a-^{13}C]Folate Binary Complex. The ^{13}C spectrum of the binary complex of [4,6,8a-^{13}C]folic acid with *L. casei* DHFR was recorded at two different pH values, and these are shown in Figure 2. Again, there is evidence for multiple conformations; five ^{13}C signals were detected, one of these corresponding to two overlapping signals. This is consistent with the presence of two conformations at pH 7.0. The ^{13}C chemical shifts of the bound species are very similar to the values observed for forms IIa and IIb in the ternary complex: the assignments were made on this basis, and these are reported in Table I. The intensities of the signals in the spectrum of the binary complex do not change markedly on lowering the pH to 5.3.

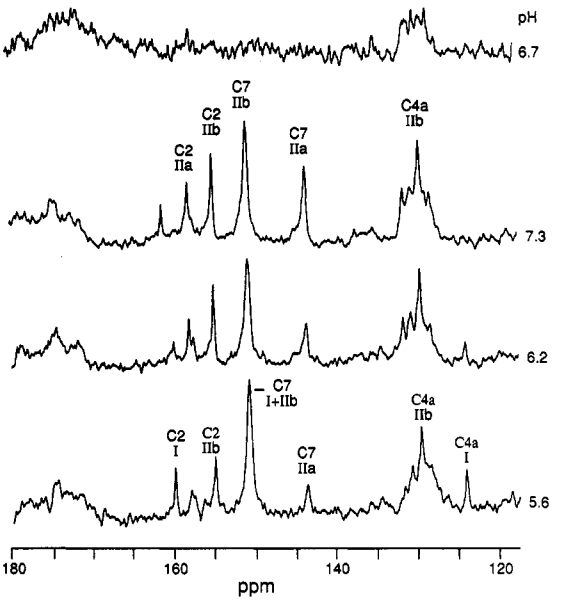


FIGURE 3: Low-field region of the 100.6-MHz ^{13}C NMR spectra at 281 K of the ternary complex of *L. casei* DHFR with NADP$^+$ and (spectra from top to bottom) unlabeled folic acid and [2,4a,7,9-^{13}C]folic acid at pH 7.3, 6.2, and 5.6, respectively.

L. casei DHFR–[2,4a,7,9-^{13}C]Folate–NADP$^+$ Ternary Complex. Figure 3 shows the low-field region of the ^{13}C spectrum of the ternary complex formed by [2,4a,7,9-^{13}C]-folic acid, NADP$^+$, and *L. casei* DHFR. Also shown in the figure is the natural abundance ^{13}C spectrum of *L. casei* DHFR complexed with NADP$^+$ and unlabeled folic acid. For the complex with the labeled folate at pH 7.3, carbons C2, C7, and C9 in the bound folate give rise to two ^{13}C signals each (the C9 signals at 45.3 and 46.1 ppm are not included in Figure 3). The signals from C4a in bound folate resonate in a spectral region that is badly overlapped by signals from the naturally occurring ^{13}C in the protein. The presence of multiple signals again confirms the presence of forms IIa and IIb at high pH values. As before, the assignments were made to forms IIa, IIb, and I by observing the signal intensity changes as a function of pH.

^{1}H–^{13}C HMQC experiments were carried out at pH 5.5 and 8.1 to connect C7 ^{13}C signals with their corresponding H7 proton chemical shift values in forms I, IIa, and IIb. This confirms the assignment of the C7 ^{13}C signals in the one-dimensional spectrum and in particular confirms that the more intense ^{13}C signal of each pair observed at high pH corresponds to form IIb where the H7 proton has a folate-like ^{1}H chemical shift and environment. Figure 4 shows the ^{1}H–^{13}C HMQC–NOESY spectrum of the DHFR-[2,4a,7,9-^{13}C]folate–NADP$^+$ complex at pH 5.5. The H7 signals appear as doublets because no ^{13}C decoupling was used. The ^{13}C cross-section for C7 in form IIa shows NOEs between folate H7 and methyl protons in Leu 27 while that in form IIb (which overlaps C7 of form I) shows a connection between H7 and the Ala 97 methyl protons. The ^{13}C cross-section of C9 (not shown) allows one to see the NOE connections between bound H9 protons and Thr 45 (CH_3) and Phe 49 (ϵ, ζ) protons and also to the benzoyl ring protons of folate.

In 500-MHz ^{1}H NMR experiments on the ternary complex at pH 5.3, a signal observed at 13.95 ppm was found to have NOE connections to the bound NADP$^+$ H4 and H5 signals and to the Leu 4 (δ_1, δ_2), Trp 5 (α, β_1, β_2), Phe 30 (δ, ϵ), and Ala 97 (α, β) signals: there is no obvious candidate protein signal which could give such effects. A possible explanation would be that the signal at 13.95 ppm arises from an enolic

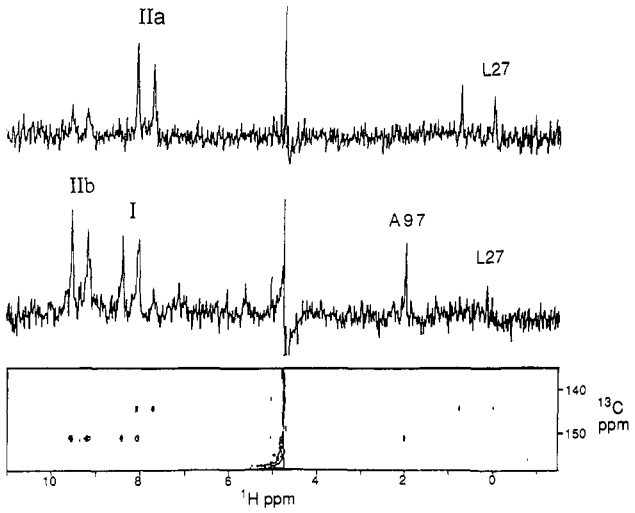


FIGURE 4: Part of the two-dimensional ^{1}H–^{13}C HMQC–NOESY spectrum of the ternary complex of *L. casei* DHFR with [2,4a,7,9-^{13}C]folic acid at 280 K and pH 5.5 showing the region connecting C7 with H7 and signals from nearby residues. The upper traces are the rows at the frequency of the C7 signal in forms I, IIa, and IIb. H7 signals appear as doublets because no ^{13}C decoupling in F_2 was used.

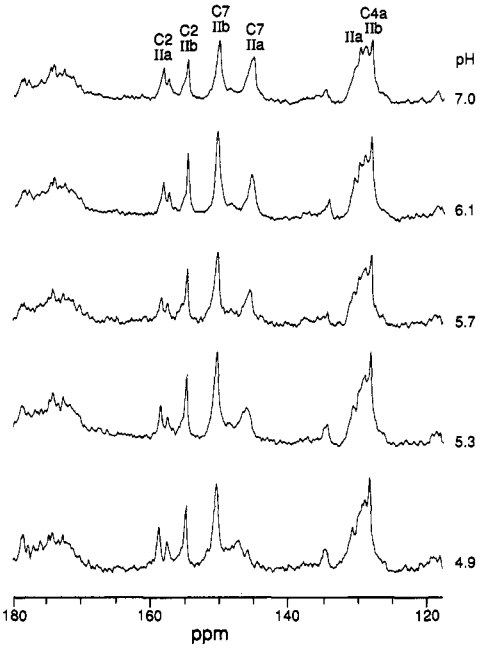


FIGURE 5: Low-field region of the 100.6-MHz ^{13}C spectra at 281 K of the binary complex of *L. casei* DHFR with [2,4a,7,9-^{13}C]folic acid at different pH values.

proton in the bound folate in form I (see later).

L. casei DHFR–[2,4a,7,9-^{13}C]Folate Binary Complex. Figure 5 shows the low-field region of the ^{13}C NMR spectra of the binary complex of DHFR with [2,4a,7,9-^{13}C]folate examined as a function of pH. The appearance of multiple signals in all the spectra for each labeled carbon confirms the presence of the two conformations of folate seen previously at the higher pH values. At pH 7.0 the two forms have ^{13}C chemical shifts similar to those observed previously for forms IIa and IIb in the ternary complex with NADP^{+}. Additional support for the ^{13}C assignments for forms IIa and IIb in the case of C7 is provided by ^{1}H–^{13}C HMQC experiments (see Figure 6): the ^{1}H assignments for H7 in forms IIa and IIb are known (8.1 and 9.3 ppm, respectively), and thus the connected C7 signals can be assigned.

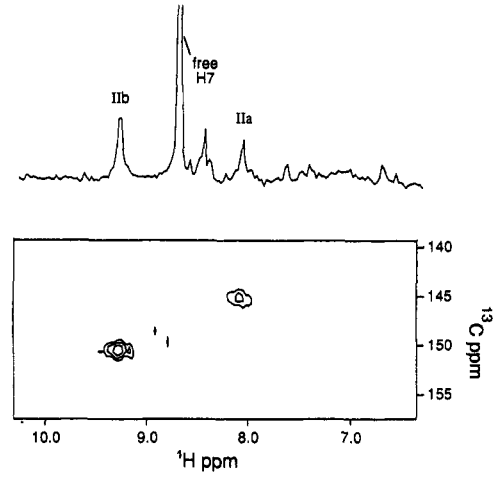


FIGURE 6: Part of the two-dimensional ^{1}H–^{13}C HMQC experiment of the binary complex of *L. casei* DHFR with [2,4a,7,9-^{13}C]folic acid at 280 K and pH 5.9 showing the region connecting C7 and H7. The upper trace is a cross-section from a 2D NOESY experiment taken at the frequency of the free H7 signal and shows the cross-peaks connecting the H7 frequencies of the exchanging free and bound folate species.

As the pH is lowered from 7.0 (Figure 5) the signal of C7 at 145.4 ppm (IIa) starts to become very broad and to shift downfield, reaching 147.0 ppm at pH 4.9. This C7 signal shows the same pH behavior even in the presence of an excess of the free enzyme or in the presence of excess folic acid, and thus the spectral changes cannot be attributed to an exchange process between bound and free folate. The most likely explanation is that the C7 signal in form IIa is in intermediate exchange with that of the C7 carbon in the bound form I. If we now turn to the ^{13}C shifts for C2, C4, and C6 in form IIa in the binary complex, it is noted that the values change with pH: at low pH values (pH 5.3) the chemical shifts are 158.6, 170.2, and 151.2 ppm, respectively, whereas at higher pH values (pH 7.0) they are 158.2, 170.0, and 150.9 ppm, respectively. This behavior could be reflecting the same exchange process between forms IIa and I. The small chemical shift differences between the C2, C4, and C6 carbons in form IIa and form I (as estimated from the ternary complex) compared with that for the C7 carbon would allow the former to show fast exchange behavior while the latter shows intermediate exchange. Thus, for the first time, we have evidence for all three forms being present in the binary complex as well as in the ternary complex, confirming that the presence of NADP^{+} is not required for these equilibria to be observed and that the origin of the effect arises from different modes of folate binding. In our earlier work usig [5-^{15}N]folate to study the binary complexes, the two bound forms observed at high pH were tentatively interpreted as arising from forms I and II (Birdsall *et al.*, 1987). However, the ^{13}C results for the binary complexes reported here clearly indicate that the two forms at high pH are forms IIa and IIb, with a small amount of form I appearing at low pH with an apparent pK < 5.0 describing the pH dependence of the relative populations of forms I and IIa + IIb.

L. casei DHFR–Methotrexate Complexes. The ^{13}C NMR spectra of the binary DHFR complexes containing either [2,-4a,6-^{13}C]- or [4,7,8a,9-^{13}C]methotrexate have also been recorded and the chemical shifts of the bound methotrexate (MTX) measured (see Table I). Each carbon gives rise to only a single resonance for bound methotrexate, confirming that the inhibitor is bound in a single conformational state in the protein (Birdsall *et al.*, 1989a) in contrast to the situation

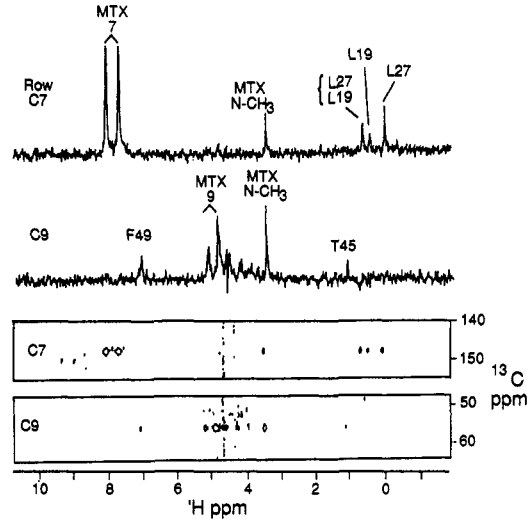


FIGURE 7: Part of the two-dimensional ^1H–^{13}C HMQC–NOESY experiment of the binary complex of *L. casei* DHFR with [4,7,-8a,9-^{13}C]methotrexate at 308 K and pH 6.5 showing the regions containing C7 and C9. The upper traces are the rows at the frequency of C7 and C9 showing the connections to their attached protons and NOEs to signals to nearby residues. The H7 signal appears as a doublet because no ^{13}C decoupling in F_2 was used.

with the *E. coli* DHFR–methotrexate complex (Falzone *et al.*, 1991; Huang *et al.*, 1991; Cheung *et al.*, 1992a). Previous ^{13}C studies (Cocco *et al.*, 1981; Birdsall *et al.*, 1989a) have shown that N1 is protonated in the bound methotrexate.

Figure 7 shows the ^1H–^{13}C HMQC–NOESY spectrum of the DHFR–[4,7,8a,9-^{13}C]methotrexate complex and the cross-sections corresponding to the C7 and C9 ^{13}C resonance frequencies: the H7 and H9 signals appear as multiplets because no ^{13}C decoupling was used in F_2. The C7 proton clearly shows connections to signals at the assigned positions for protons in the methyl groups in Leu 19 and Leu 27 as well as to those in the $N_{10}CH_3$ group of bound methotrexate. The C9 protons show connections to Thr 45 (CH_3) and Phe 49 (ϵ, ζ) and an intramolecular NOE to $N_{10}CH_3$ of methotrexate. No connections were seen between H9 and the benzoyl ring protons probably because the latter have broad signals due to slow ring flipping.

Estimation of ^{13}C Chemical Shifts for the Enol Form of Folic Acid

In this work ^{13}C chemical shift data for bound folate are used to assign the bound tautomeric forms. Because ^{13}C chemical shift data for the enolic state of folic acid are not directly accessible (the enolic state does not exist in free solution), estimates of these shifts were sought from studies of model compounds known to exist in the enolic form.

From 2-Amino-4-methoxy-6-methylpteridine Data. We have measured the ^{13}C chemical shifts of 2-amino-4-methoxy-6-methylpteridine (Table I) and then estimated the substituent ^{13}C shielding effects of replacing a 4-OMe group by a 4-OH group in the pteridine ring using ^{13}C data from substituted aromatic compounds as described below.

The shift changes on replacing a OMe with a OH group in monosubstituted benzenes are the following: for the directly substituted carbon, –4.5 ppm; ortho, 1.7 ppm; meta, 0.4 ppm (Levy *et al.*, 1980; Ewing, 1979). However, the inductive effect at the substituted carbon is expected to be smaller in a 2-substituted pyridine or a 4-substituted pyrimidine due to competition for electronic charge between the ring nitrogen and the electron-withdrawing substituent (Craik, 1983; Turner & Cheeseman, 1976; Cheeseman *et al.*, 1979). For example, an $NH_2 \rightarrow$ OMe substitution in a monosubstituted benzene causes a substituent shift at C1 of 13.4 ppm (Levy *et al.*, 1980; Craik, 1983) compared with a corresponding substituent shift of only 4.4 ppm in a 2-substituted pyridine (Stothers *et al.*, 1972) and 4.4 ppm in 4-substituted 2-amino-6-methylpteridine (unpublished results). On the basis of this, an estimate of the substituent shift contribution for an OMe → OH substitution in a 4-substituted pteridine would be –1.4 ppm (that is, 32% of the benzene substituent effect) for the directly substituted carbon. In contrast, the substituent shifts at carbons ortho or meta to the substituent for a 4-substituted pteridine are more similar to those for a monosubstituted benzene (Stothers, 1972, and data in Table I).

Estimates for the ^{13}C chemical shifts in the hypothetical 2-amino-4-hydroxy-6-methylpteridine were thus obtained by using the measured ^{13}C shift data for 2-amino-4-methoxy-6-methylpteridine in combination with the following OMe → OH substituent effects: substituted carbon, –1.4 ppm; ortho, 1.7 ppm; meta, 0.4 ppm. Estimates were obtained for the enol form with and without protonation of N1 by starting from the measured data for 2-amino-4-methoxy-6-methylpteridine obtained for the protonated and nonprotonated species (see Table I).

From DHFR–Methotrexate Data. Estimates of the ^{13}C chemical shifts for the enol form of folate bound to DHFR in the methotrexate-like orientation were also obtained from data measured on the DHFR–methotrexate complex after allowing for the shielding effects for the $NH_2 \rightarrow$ OH substitution which were estimated to be the following: substituted carbon, 3.1 ppm; ortho, 2.4 ppm; meta, –1.2 and 1.5 ppm (equivalent to C2 and C8a, respectively). The above substituent effects were derived as follows: the ^{13}C chemical shifts of the hypothetical 4-hydroxypyrimidine were estimated as above from the measured data of 4-ethoxypyrimidine (Cheeseman *et al.*, 1979), with correction for small shift changes upon replacement of an aromatic OEt group by a OMe one (Breitmaier *et al.*, 1975). This calculated shift is then compared with the measured data of 4-aminopyrimidine (Cheeseman *et al.*, 1979) to give the estimated effects for $NH_2 \rightarrow$ OH substitution. The $NH_2 \rightarrow O^-$ substituent effects were estimated directly from those for 4-substituted pyrimidines (Cheeseman *et al.*, 1979) to be the following: substituted carbon, 7.5 ppm; ortho, 8.9 ppm; meta, –3.5 (C2) and –1.7 ppm (C8a).

By using the model compound data derived from bound methotrexate (N1 protonated), some allowance is included for shielding contributions resulting from complex formation. This model provides the ^{13}C data for the enol in the N1 protonated form. The data for the enol in the N1 unprotonated form were estimated by using the protonation data from the 2-amino-4-methoxy-6-methylpteridine (see Table I).

^1H Chemical Shift of Protein Residues in the Pteridine Ring Binding Site

2D COSY, HOHAHA, and NOESY and 3D HOHAHA–HMQC and NOESY–HMQC spectra for the ternary complexes DHFR–folate–$NADP^+$ have been recorded and analyzed (pH 5.3 and 7.2). Many sequence-specific assignments (>90% for the pH 5.3 sample) have been made and compared with assignments obtained for the DHFR–methotrexate–$NADP^+$ complex using similar procedures. Table II gives a list of assigned ^1H resonances from some of the protein residues in the pteridine ring binding site and the chemical shift

Table II: *Differences between the Chemical Shifts for DHFR Protons near the Pteridine Protonated N1 and 2NH$_2$ Group in the Folate–NADP$^+$ (Form I) and MTX–NADP$^+$ Complexes*

		^{1}H chemical shift[a]		
residue	proton	DHFR–folate–NADP$^+$ (I)	DHFR–MTX–NADP$^+$	difference
Ala 6	α	5.61	5.61	0.00
	β	1.44	1.52	–0.08
Trp 21	ϵ1	10.13	10.21	–0.08
Asp 26	N	7.00	6.99	0.01
	α	5.39	5.35	0.04
	β	2.27	2.27	0.00
Leu 27	α	3.90	3.87	0.03
	δ2	–0.09	–0.01	–0.08
Tyr 29	δ	6.81	6.80	0.01
	ϵ	6.70	6.70	0.00
Phe 30	N	9.09	9.13	–0.04
Thr 116	β	3.98	4.02	–0.04
	γ	0.55	0.55	0.00
Tyr 155	δ	6.54	6.52	0.02
	ϵ	6.54	6.59	–0.05

[a] Chemical shifts measured in parts per million from DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate).

differences between form I of the DHFR–folate–NADP$^+$ complex (pH 5.3) and the DHFR–methotrexate–NADP$^+$ complex. The chemical shifts for protein protons close to the methotrexate N1 binding site provide particularly useful information. For example, in spectra recorded at pH 5.3 the distinctive and unusual chemical shifts of the α-CH and NH protons of Asp 26 (1.01 and 1.41 ppm upfield from random coil values, respectively) are very similar in the folate (form I) and methotrexate ternary complexes: this points to the folate binding in an N1 protonated form to Asp 26 in a manner similar to that found for methotrexate (Bolin *et al.*, 1982; Cocco *et al.*, 1981; Birdsall *et al.*, 1989a). The chemical shifts of the N1(ϵ) signal from Trp 21 in forms I and IIa of the folate ternary complex (10.13 and 10.24 ppm, respectively) are similar to that in the methotrexate ternary complex (10.21 ppm) and approximately 0.7 ppm different from that in form IIb of the folate complex (10.98 ppm). In the methotrexate complex, the Trp 21 N1(ϵ) proton is expected to form part of a hydrogen-bonded network involving a bridging water molecule (Wat 253), Asp 26, and the methotrexate N1 proton (Bolin *et al.*, 1982): thus, the above chemical shift data are consistent with folate in forms I and IIa binding to DHFR in an N1 protonated form. The chemical shift differences for protons in other residues close to the methotrexate N1 binding site (for example, Ala 6, Leu 27, and Phe 30) are also fairly small as are those in residues near the methotrexate 2-NH$_2$ group binding site (Tyr 29, Thr 116, and Tyr 155).

DISCUSSION

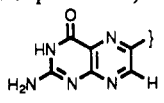
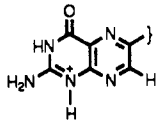
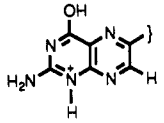
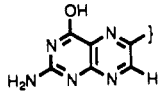
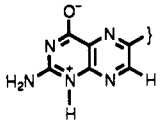
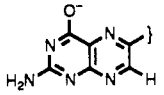
The observation of multiple ^{13}C signals for all of the labeled carbons in the pteridine ring of folate bound to *L. casei* DHFR confirms the presence of the multiple conformations detected earlier (Birdsall *et al.*, 1981, 1982, 1987, 1989b). The observed changes in signal intensities as a function of pH confirm the pH dependence of the three forms of the complex (forms I, IIa, and IIb) in the ternary complex with NADP$^+$ and allow two forms corresponding to IIa and IIb to be identified in the binary complex. Furthermore, consideration of an intermediate exchange phenomenon in the binary complex leads to the detection of a third form corresponding to form I. Detailed information about the orientation and tautomeric states of the pteridine ring in the different forms can be determined.

Orientation of the Pteridine Ring in Different Complexes. In previous studies the different conformations have been characterized in terms of the orientation of the pteridine ring in the binding site. From NOESY experiments it was possible to show that forms I and IIa had the pteridine ring in a methotrexate-like orientation by establishing NOE connections between the pteridine H7 proton and the assigned methyl protons of Leu 27. The latter NOEs had previously been detected in NOESY spectra of DHFR complexes with methotrexae (Birdsall *et al.*, 1989a) and are those expected from consideration of the crystal structure studies of Bolin and co-workers (1982). The ^{1}H–^{13}C HMQC–NOESY experiments described here can also be used to characterize the environments of the pteridine ring in the different folate conformations. These experiments not only confirm the earlier work showing the pteridine ring in a methotrexate-like orientation in forms I and IIa but also provide structural evidence that the pteridine ring of folate in form IIb has the orientation expected for the active folate complex, that is, turned over with respect to the methotrexate orientation. In the ^{1}H–^{13}C HMQC–NOESY spectrum of DHFR-[2,4a,7,9-^{13}C]folate–NADP$^+$ (Figure 4), the H7 proton in form IIa shows an NOE connection to the methyl protons of Leu 27 (a similar connection is seen in the ^{1}H–^{13}C HMQC–NOESY spectrum of the methotrexate complex (Figure 7)). The H7 proton in form IIb shows a connection with the Ala 97 methyl proton signal, indicating the proximity of these protons. Consideration of the crystal structure data of Bolin and co-workers (1982) for the *L. casei* DHFR–MTX–NADPH complex indicates that a "turned over" pteridine ring occupying essentially the same binding site would have its H7 proton ~3 Å from the Ala 97 methyl protons. One-dimensional NOE difference spectra selectively irradiating the H7 proton signals in forms I, IIa, and IIb for the DHFR–folate–NADP$^+$ complex at pH 6.5 confirmed that only form IIb gives a large NOE to Ala 97 CH_3 (spectra not shown).

Tautomeric States of Folate in Its Bound Forms. Information on the tautomeric state of bound folate can be obtained by considering the ^{13}C chemical shift data summarized in Table I. If we consider form IIb in the binary and ternary complexes of DHFR, the ^{13}C chemical shifts of the C4, C2, and C4a carbons are noted to be similar to the corresponding values for free folic acid at pH 5.5 (see Table I). Since these carbon shieldings are sensitive to the keto/enol equilibrium involving C4 and N3, then the similarity of the C4, C2, and C4a ^{13}C shifts indicates that bound folate in state IIb is in the same tautomeric form as free folic acid at pH 5.5, for which the structure is known to be in the 4-keto form with N1 unprotonated (Blakley, 1969) (see structure **3** in Table III). In contrast, the ^{13}C chemical shifts of the C4 and C2 carbons of bound folate in form IIa in the binary and ternary complexes are different from those in form IIb, suggesting that there has been a change in the keto/enol tautomeric and/or protonation states of N1 and N3. In order to determine these states in the bound folate species, we have compared the observed bound chemical shifts with those of the "model" species (see Table I and text), focusing our attention on the ^{13}C chemical shift data for the pyrimidine ring. To facilitate the comparisons, we have tabulated the ^{13}C chemical shift differences between corresponding carbons in the bound and model compound species (see Table III) and calculated values for $\Sigma\Delta^2/4$, the average of the sum of the squares of the chemical shift differences for the four carbons considered. The size of this term can be used as a measure of the goodness of fit between the bound and model species.

It is seen that for form IIb (binary and ternary) the smallest $\Sigma\Delta^2/4$ values are observed for the keto (N1 unprotonated)

Table III: ^{13}C Chemical Shift Differences (ppm) between Folate in *L. casei* DHFR Complexes and Model Compounds in Various Tautomeric and Ionization Forms

model	form	C4	C8a	C2	C4a	$\Sigma\Delta^2/4$
keto (N1 unprotonated)	IIb (binary)	−1.6	3.2	−0.9	−0.4	3.4
	IIb (ternary)	−2.5	4.1	−0.7	1.0	6.1
3 	IIa (binary)	4.1	−6.9	2.6	1.5 ± 2	18.4
	IIa (ternary)	3.0	−7.4	2.3	1.5 ± 2	17.8
	I (ternary)	5.4	−3.5	4.2	−4.6	20.0
keto (N1 protonated)	IIb (binary)	5.5	10.6	2.6	0.3	37.4
	IIb (ternary)	4.6	11.5	2.8	1.7	41.0
4 	IIa (binary)	11.2	0.5	6.1	2.2 ● 2	41.9
	IIa (ternary)	10.1	0	5.8	2.2 ± 2	35.1
	I (ternary)	12.5	3.9	7.7	−3.9	61.5
enol (N1 protonated)[a]	IIb (binary)	−3.5	10.7	−1.8	1.7	33.2 (35.8)[b]
	IIb (ternary)	−4.4	10.6	−1.8	3.8	37.4 (45.5)
5 	IIa (binary)	2.2	0.6	1.7	3.6 ± 2	5.3 (11.7)
	IIa (ternary)	1.1	−0.9	1.2	4.3 ± 2	5.5 (10.1)
	I (ternary)	3.5	3.0	3.1	−1.8	8.5 (10.8)
enol (N1 unprotonated)[a]	IIb (binary)	−3.8	2.1	−6.6	2.4	17.0 (20.6)[b]
	IIb (ternary)	−4.7	2.0	−6.6	4.5	22.5 (26.6)
6 	IIa (binary)	1.9	−8.0	−3.1	4.3 ± 2	23.9 (31.3)
	IIa (ternary)	0.8	−9.5	−3.6	5.0 ± 2	32.2 (32.8)
	I (ternary)	3.2	−5.6	−1.7	−1.1	11.4 (9.6)
enolate (N1 protonated)[a]	IIb (binary)	−7.9	13.8	0.5	−4.8	69.0
	IIb (ternary)	−8.8	13.7	0.5	−2.7	68.2
7 	IIa (binary)	−2.2	3.7	4.0	−2.9 ± 2	10.7
	IIa (ternary)	−3.3	2.2	3.5	−2.2 ± 2	8.2
	I (ternary)	−0.9	6.1	5.4	−8.3	34.0
enolate (N1 unprotonated)[a]	IIb (binary)	−8.2	5.2	−4.3	−4.1	32.4
	IIb (ternary)	−9.1	5.1	−4.3	−2.0	32.8
8 	IIa (binary)	−2.5	−4.9	−0.8	−2.2 ± 2	8.9
	IIa (ternary)	−3.6	−6.4	−1.3	−1.5 ± 2	14.5
	I (ternary)	−1.2	−2.5	0.6	−7.6	16.4

[a] Enol and enolate model shifts estimated from data for methotrexate with N1 protonated bound to DHFR (in binary and ternary complexes) and allowing for substituent effects $NH_2 \rightarrow OH$ or $NH_2 \rightarrow O^-$ (see text). Effects of N1 deprotonation estimated from data for 2-amino-4-methoxy-6-methylpteridine obtained in neutral and acid solutions. [b] The numbers in parentheses are $\Sigma\Delta^2/4$ values calculated using the enol model data calculated from the data for 2-amino-4-methoxy-6-methylpteridine (see text).

form (structure **3**). It is clear that neither the keto (N1 protonated) form (structure **4**) nor any of the enolic forms (structures **5**–**8**) would fit the data. In contrast, the data for form IIa do not fit the keto (N1 unprotonated) model (structure **3**) but show a reasonable fit with several of the enolic models (structures **5**, **7**, and **8**). While it is not possible to differentiate between these structures on the basis of the ^{13}C chemical shift data, the ^{1}H studies discussed earlier suggest an N1 protonated form (structure **5** or **7**). Future proposed NMR studies on enzyme complexes formed with ^{15}N-labeled folic acids should resolve some of these ambiguities.

Reasonable fits of the data for bound form I in the ternary complex are given by two of the enol models (structures **5** and **6**). Thus, the ^{13}C shift data do not allow us to distinguish between the N1 protonated and unprotonated forms. However, it was seen earlier that comparison of ^{1}H chemical shifts for Asp 26 in the folate and methotrexate ternary complexes suggest that folate is protonated at N1 in form I. In this form (structure **5**), the folate pteridine ring could make interactions with the protein very similar to those observed in the DHFR–methotrexate–NADPH complex (Bolin *et al.*, 1982); this is consistent with the similar chemical shifts seen for residues in the binding site of the two complexes. Some additional evidence for the form I folate species being an enol is provided by the previously mentioned low-field ^{1}H signal (13.95 ppm) havine the NOEs expected for an enol proton at that position on the pteridine ring.

It is noted that form IIb (structure **3**) has one proton less than form I (structure **5**), which would offer a simple explanation for the observed pH-dependent conformational equilibrium. One of the possible structures for form IIa would also fit this explanation (structure **7**).

To summarize, our previous studies have shown that there are three interconverting conformational forms of the complex *L. casei* DHFR–folate–NADP$^+$: forms I, IIa, and IIb, with form I predominantly at low pH (Birdsall *et al.*, 1989b). The present work shows that form IIb, the catalytically active conformation, has the pteridine ring turned over by 180° as compared to the methotrexate–DHFR complex, whereas forms I and IIa have a pteridine ring orientation similar to that in the methotrexate–DHFR complex. The present study indicates that the catalytically active structure, form IIb, is in the 4-keto form (the same as free folate) with N1 unprotonated whereas form I and IIa are in enolic forms.

The complexes described here are examples of where a flexible ligand can bind in several conformational states to an enzyme. It is important to characterize these different states in terms of their electronic and conformational structures since each state could provide a starting point for the design of enzyme inhibitors.

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