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^{15}N and ^{1}H NMR evidence for multiple conformations of the complex of dihydrofolate reductase with its substrate, folate

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The binding of folate to *Lactobacillus casei* dihydrofolate reductase in the presence and absence of $NADP^+$ has been studied by ^{15}N NMR, using [5-^{15}N]folate. In the presence of $NADP^+$, three separate signals were observed for the single ^{15}N atom, in agreement with our earlier evidence from ^{1}H and ^{13}C NMR for multiple conformations of this complex [(1982) Biochemistry 21, 5831–5838]. The ^{15}N spectra of the binary enzyme-folate complex provide evidence for the first time that this complex also exists in at least two conformational states. This is confirmed by the observation of two separate resonances for the 7-proton of bound folate, located by two-dimensional exchange spectroscopy.

Dihydrofolate reductase; Folate; ^{15}N-NMR; 2D NMR

1. INTRODUCTION

Dihydrofolate reductase is an NADPH-linked dehydrogenase which catalyses the reduction of folate and dihydrofolate to tetrahydrofolate. It is the site of action of the important 'anti-folate' drugs, including methotrexate and trimethoprim. In recent years, a substantial body of structural information on complexes of the enzyme with inhibitors has become available from X-ray crystallography and NMR spectroscopy (reviews [1–4]). By contrast, much less structural information is available on complexes with substrates, although stereochemical studies have established that the substrate must bind in a different orientation to that seen for methotrexate in the crystal [5–7].

We have previously studied the dihydrofolate reductase-folate-$NADP^+$ complex using ^{1}H, ^{13}C and ^{31}P NMR [8] and shown that this complex exists in solution in multiple conformations. The ^{13}C NMR spectrum of the enzyme complex in which the 3-carboxamido of $NADP^+$ was selectively enriched with ^{13}C clearly showed signals from three conformational states, called forms I, IIa and IIb, which are in slow exchange with each other on the NMR time scale. Forms IIa and IIb, which always appear in constant relative proportions, are the predominant species at high pH whereas form I appears to be the single predominant form at low pH.

Evidence for multiple conformations is also seen in the ^{1}H resonances of the nicotinamide ring, but not in the adenine ^{1}H signals or in the ^{31}P signals

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of the coenzyme. We concluded that the differences between the three conformations are localised to the region around the nicotinamide ring, i.e. close to the bound substrate molecule. Most of the available information on this conformational equilibrium has come from resonances of the coenzyme; there is only one carbon-bound proton on the pteridine ring of folate, namely H7, and in our earlier studies [8] it was not possible to locate this resonance in all the states of the complex. In order to establish how folate is affected by this equilibrium and whether the existence of the equilibrium depends on the presence of the coenzyme, we have now studied folate binding by ^{15}N and two-dimensional ^{1}H NMR.

2. MATERIALS AND METHODS

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R [9]. [5-^{15}N]Folate was prepared from 2,6-diamino-4-pyrimidinol by nitrosation using $Na^{15}NO_2$ and reduction of the 5-nitroso compound with $Na_2S_2O_4$ to yield triamino-4-pyrimidinol. Condensation with 2-bromo-3,3-dimethoxypropionaldehyde, followed by acetylation and hydrolysis of the acetal with 88% HCOOH [10] gave the 2-[5-^{15}N]acetamidopterin-6-aldehyde. The aldehyde was reductively condensed with diethyl *p*-aminobenzoyl-L-glutamate in the presence of borane-dimethylamine complex in glacial HOAc solution via the general method of Plante [11] to afford the 2-acetamido diethyl ester of the labeled folate in 28% yield. This intermediate was purified by chromatography on silica gel with elution by CH_3OH:CH_2Cl_2 (1:9). TLC analysis (silica gel, same solvent) showed a single UV-absorbing spot at R_f 0.4; mass spectrum (unlabeled run), *m/e* 539; NMR of unlabeled material ($CDCl_3$) δ 1.30 (6H, t, ester CH_3), 2.40 (7H, m, CH_3CO,-CH_2CH_2-glutamate), 4.20 (4H, m, -O$\underline{CH_2}CH_3$), 4.75 (3H, br s, C_9-CH_2, CHCO of glutamate), 6.70 (3H, m, 3′,5′-ArH, NH), 7.40 (1H, d, NH), 7.75 (2H, d, 2′,6′-ArH), 8.95 (1H, s, C-7H).

Subsequent saponification of the diester with 1 N NaOH in aqueous CH_3OH yielded [5-^{15}N]folic acid; UV λ pH 13 256 nm (log ϵ 4.40), 286 (4.39), 366 (3.95), identical with authentic folic acid standard. The HPLC spectrum run on a Waters C_{18}-Radial Pak A cartridge with elution by CH_3OH:0.1 N NaH_2PO_4 (pH 6.7), 1:3, showed it to be 99% pure and identical to the folic acid standard in retention time (with admixture) and peak height as measured by absorbance at 3 different wavelengths, 254, 284 and 360 nm.

500 MHz ^{1}H NMR spectra were obtained by using a Bruker AM500 spectrometer. The 0.4 ml samples contained 1.0 mM enzyme in 50 mM potassium phosphate, 100 mM KCl, 1 mM EDTA, 1 mM dioxan with 2.0 molar equivalents of folate in $^{2}H_2O$. The two-dimensional exchange experiment was carried out in the phase-sensitive mode using the NOESY $(90–t_1–90–\tau_m–90–t_2–d)_n$ sequence and the method of time-proportional phase incrementation [12]. 1024 data points were recorded in t_2 for each of 233 t_1 values. A spectral width of 6410 Hz was used with the carrier frequency at the centre of the spectrum and quadrature detection in both dimensions. The relaxation delay (d) was 0.6 s, and the mixing time (τ_m) was 100 ms. The number of scans per FID (n) was 256 and the total accumulation time for the experiment was 19 h. The data were zero filled to 1024 points in t_1 and processed with a sine bell function in each dimension, shifted $\pi/32$ in t_2 and $\pi/16$ in t_1. Two-dimensional Fourier transformation produced a spectrum with a digital resolution in the F_2 dimension of 12.5 Hz/pt.

The ^{15}N NMR spectra were obtained from 3.0 ml samples containing 3.9 mM enzyme in 50 mM potassium phosphate, 100 mM KCl in $^{2}H_2O$, with 0.8 molar equivalent [5-^{15}N]folate. 1.0 molar equivalent of $NADP^+$ was added later to form the ternary complex. The pH* of the sample was adjusted between pH* 5.2 and 7.2 using 1.0 M solutions of ^{2}HCl or $NaO^{2}H$. (pH* indicates a meter reading uncorrected for the deuterium isotope effect on the glass electrode.)

20 MHz ^{15}N NMR spectra were obtained using a Bruker WM200 spectrometer. Quadrature detection was used with a spectral width of 5 kHz, a pulse interval of 1.64 s, and a 60–90° pulse. Spectra were recorded for approx. 24 h averaging 48000 transients. A line broadening of 2–5 Hz was applied before transformation. No ^{1}H decoupling was used.

$^{15}NH_4Cl$ (0.1 M in 0.1 M HCl containing 10% $^{2}H_2O$) was used as an external reference; its resonance appears 295.1 ppm upfield from that of free [5-^{15}N]folate at pH* 6.3.

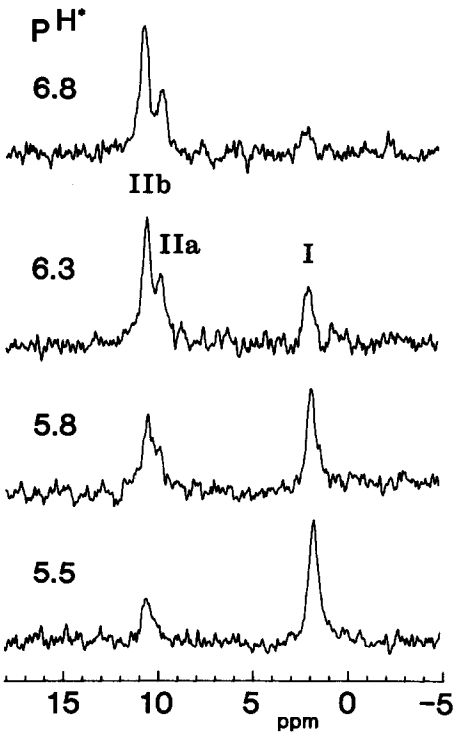

Fig.1. 20 MHz ^{15}N NMR spectra at 278 K of the dihydrofolate reductase-[5-^{15}N]folate-NADP$^+$ complex at a series of pH* values.

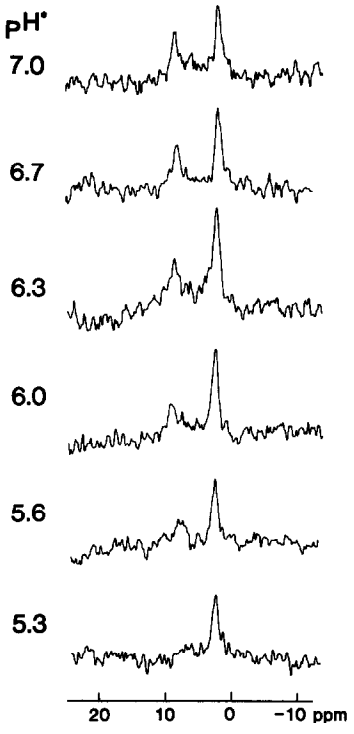

Fig.2. 20 MHz ^{15}N NMR spectra at 278 K of the dihydrofolate reductase-[5-^{15}N]folate complex at a series of pH* values.

3. RESULTS AND DISCUSSION

The sample used for the ^{15}N experiments contained 3.9 mM enzyme and 3.1 mM [5-^{15}N]folate. Under the conditions of the experiment, the binding constant of folate is 2.7×10^5 M^{-1} (B. Birdsall, unpublished) for the binary complex and approx. 20-fold higher in the presence of NADP$^+$ [8]. It follows that the concentration of free folate will be negligibly small, and any ^{15}N resonances observed will arise from bound [5-^{15}N]folate.

Fig.1 shows the 20 MHz ^{15}N spectra of the ternary complex, dihydrofolate reductase-[5-^{15}N]folate-NADP$^+$, at a series of pH* values. In the spectrum recorded at pH* 6.3, three signals are observed from the single enriched ^{15}N atom of bound folate. This shows clearly that the complex exists in solution in three conformational states. As the pH* is changed, the chemical shifts of the ^{15}N resonances are unaffected, but their relative intensities change. The pattern of intensity changes is closely similar to that reported previously [8] in the ^{13}C spectra of the same complex formed with [*carboxamide*-^{13}C]NADP$^+$. This allows us to assign the three ^{15}N signals to the three conformational states identified earlier [8]. Conformations IIa and IIb, whose ratio is independent of pH*,

Table 1

^{15}N chemical shifts of [5-^{15}N]folate bound to dihydrofolate reductase[a]

Complex	Conformation		
	I	II	
Enzyme-folate	2.7	9.1	
		IIa	IIb
Enzyme-folate-NADP$^+$	2.5	9.9	10.7

[a] In ppm downfield from the resonance of free [5-^{15}N]folate at pH* 6.3

predominate at high pH*, while conformation I predominates at low pH*. The ^{15}N chemical shifts of the three conformations of the complex are given in table 1. In all three, the resonance is downfield from that of free folate – in the case of form IIb, by more than 10 ppm.

In the ^{15}N spectra of the binary enzyme-[5-^{15}N]folate complex (fig.2), two resonances are observed from the single ^{15}N at pH* values of 5.6 or greater. Clearly this complex also exists in more than one conformational state. Comparison of the ^{15}N chemical shifts (table 1) and the pH* dependence of signal intensities suggests that the conformations seen in the binary complex are related to those seen in the ternary complex.

The ^{15}N spectrum of the binary complex at pH* 5.3 shows a single resonance at 2.7 ppm, close to the position of the ^{15}N resonance of the low-pH* form (form I) of the ternary complex. As the pH* is increased, a second resonance appears at 9.1 ppm in the spectrum of the binary complex, and increases in intensity at the expense of the peak at 2.7 ppm. The chemical shift of this signal is similar to that of the two high-pH* forms (IIa and IIb) of the ternary complex. The large difference in ^{15}N chemical shift between the low-pH* and high-pH* forms (6.4–8.2 ppm) may reflect differences in hydrogen-bonding to N5 of folate. Shift differences of this magnitude and direction could be produced if in conformation I, N5 is hydrogen-bonded (thus having a chemical shift fairly close to that in free folate, where it will presumably be hydrogen-bonded to water) but in form(s) II it is not hydrogen-bonded [13–15].

In addition to the similarities between the ^{15}N spectra of the binary and ternary complexes, there are also clear differences. Only a single ^{15}N resonance is observed at 9–10 ppm in the spectrum of the binary complex, whereas two signals (IIa and IIb) are seen in this region for the ternary complex. This could reflect a genuine difference in the conformational equilibria between the binary and ternary complexes. Alternatively, it is possible that there are three forms of the binary, as of the ternary, complex, but that the ^{15}N chemical shifts and/or interconversion rates of forms IIa and IIb of the binary complex are such that they are in fast exchange on the NMR time scale and so separate resonances are not observed. The available data and the intrinsic complexity of multisite exchange

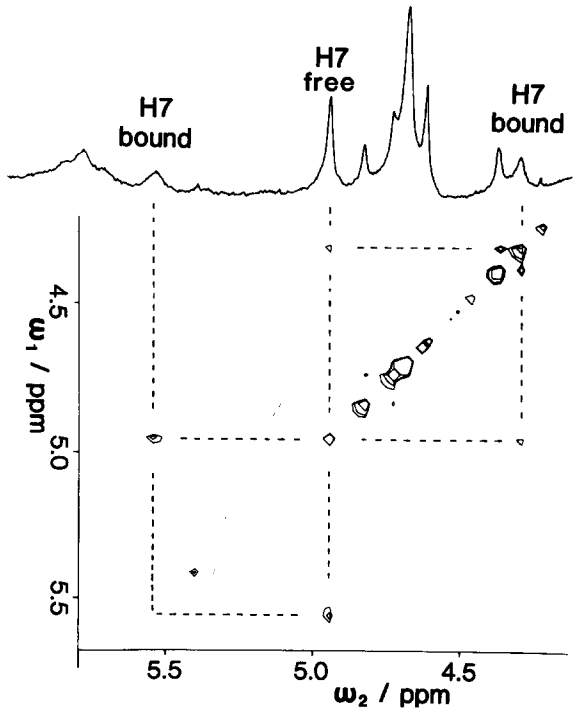

Fig.3. (Top) Low-field region of the 500 MHz ^{1}H NMR spectrum at 278 K of a sample containing dihydrofolate reductase and folate in a ratio of 1:2. The 7-proton resonances of free and bound folate are indicated. (Bottom) The corresponding region of the 2D exchange spectrum, showing the cross-peaks linking the 7-proton resonances of free and bound folate.

systems do not allow us to choose between these models. A clear-cut difference between the binary and ternary complexes is seen in the apparent pK value describing the pH* dependence of the relative signal intensities, which is 6.0 in the ternary complex [8], but >7 in the binary (fig.2).

These differences may thus be quantitative rather than qualitative, and overall the ^{15}N spectra strongly suggest that the conformations in the equilibria are the same or closely similar for the binary and ternary complexes. This indicates that the presence of the coenzyme is not required for this equilibrium to be observed, and focuses attention instead on the binding of folate.

In our previous ^{1}H studies of the enzyme-folate binary complex we have always obtained poorly resolved spectra. For example, the histidine resonances as well as those of bound and free

ligand are all broad in spectra of this complex, presumably due to exchange processes. By reducing the KCl concentration from 500 to 100 mM we have increased the association constant of folate for the enzyme from 2×10^4 to 2.7×10^5 M^{-1} (B. Birdsall, unpublished) and perturbed the rate constants in a favourable manner resulting in the sharpening of many signals in the ^1H NMR spectrum (fig.3, top). Under these conditions it was then possible to perform a successful two-dimensional NOESY/exchange experiment. This was carried out on a sample containing 2 molar equivalents of folate; the 7-proton resonance of free folate can be seen at 4.95 ppm. The cross-peaks (off-diagonal peaks) from this resonance arise from exchange of folate between the free and bound states. As can be seen in fig.3, there are two such cross-peaks, indicating that there are two bound states for the folate molecule, with 7-proton resonances at 4.4 and 5.6 ppm, respectively. Thus this experiment provides further evidence for the existence of a conformational equilibrium in the enzyme-folate binary complex.

The 1.2 ppm difference between the chemical shifts of the folate 7-proton in the two bound states suggests a considerable difference in the conformation of the bound folate in the two forms. Calculations show that the ring current contribution to the shift of H7 due to the *p*-aminobenzoyl ring of folate can vary between large positive and large negative values depending on the particular torsion angles of the bonds connecting the pteridine and *p*-aminobenzoyl rings. The large difference in shift of H7 between the two bound conformations could therefore reflect a different orientation of the pteridine ring with respect to the benzoyl ring in the two forms. If this change in orientation of the pteridine ring was accompanied by a change in hydrogen-bonding to the pteridine N5, it would also, as discussed above, explain the ^{15}N shift differences. NOE experiments designed to test this hypothesis are in progress.

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