³H-n.m.r. studies of multiple conformations and dynamic processes in complexes of folate and methotrexate with *Lactobacillus casei* dihydrofolate reductase

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[7,3',5'-³H₃]- and [7,9-³H₃]-folic acid and [7,3',5'-³H₃]-methotrexate (MTX) have been prepared and ³H-n.m.r. spectra obtained for their complexes with *Lactobacillus casei* dihydrofolate reductase (DHFR). The ³H results confirm the presence of three pH-dependent different conformational forms in the complex DHFR·NADP⁺·folate. The folate benzoyl ring could be shown to be in essentially the same environment in the

different forms, with the major differences being associated with the pterin ring. The appearance of a single resonance for the 3',5'-tritons showed that the benzoyl ring is flipping rapidly in all three forms. In contrast, the MTX complex was shown to exist as a single conformational state with the benzoyl ring flipping rate being too low to give a single averaged signal for the 3',5'-nuclei over the temperature range 283–313 K.

INTRODUCTION

Dihydrofolate reductase (DHFR) catalyses the reduction of dihydrofolate (and folate) to tetrahydrofolate using NADPH as a coenzyme [1]. The enzyme has an essential role in maintaining cellular pools of tetrahydrofolates required for the growth of the cell. There is considerable pharmacological interest in the enzyme, because it is the target for antifolate drugs such as methotrexate (MTX; 1), pyrimethamine and trimethoprim, which act by inhibiting the enzyme in malignant or parasitic cells. X-ray [2–4] and n.m.r. [5–8] structural studies on several complexes of DHFR formed with substrates and substrate analogues have been carried out with the aim of understanding the specificity of the ligand binding.

(1) Methotrexate

(2) Folic acid

Although MTX is a close structural analogue of the substrate folate (2), previous X-ray and n.m.r. stereochemical studies have shown that they bind in different ways to the enzyme. The two molecules occupy essentially the same binding site in the enzyme, but the pteridine ring of MTX has been shown to be turned over by ~180° compared with its orientation in the enzymically

productive form of the DHFR·folate complex [9–12]. More recent studies based on ¹H-, ¹⁵N- and ¹³C-n.m.r. experiments with labelled folates have shown that, in fact, folate can bind in more than one conformation to the *Lactobacillus casei* DHFR, whereas MTX binds in a single conformation [13–14]. Three different conformational states of the DHFR·folate complex have been detected, and their pH-dependence has been characterized. Two of the forms are similar (Forms I and IIa) and have conformations in which the pteridine ring is the same as that found in the DHFR·MTX·NADPH complex, while a third form (Form IIb) has its pteridine ring orientation turned over by ~180° in the form expected for a productive folate complex [13]. The use of ¹³C-selectively-enriched folates has provided a direct way of monitoring the different conformational states, whereas experiments using ¹H n.m.r. are complicated by severe spectral overlap in the ¹H spectrum of the enzyme·folate complex [14]. The present work explores the potential of ³H n.m.r. for studying these multiconformational states. By using specifically ³H-labelled ligands it should be possible to detect directly the signals from the ³H-labelled sites in the bound ligand.

The use of specifically ³H-labelled ligands could also offer some advantages in studies of dynamic processes in complexes. This can be illustrated by reference to the symmetrically substituted benzoyl rings in bound folate or MTX, which have the potential for 'ring-flipping' [15]. If the flipping is fast on the chemical-shift timescale, then a single averaged signal will be seen for H-3' and H-5' (also for H-2' and H-6'), whereas slow flipping can give rise to two separate signals for each pair of protons. Because of the signal-overlap problem and the potential for multiple conformations, such effects are sometimes difficult to monitor in ¹H spectra. In principle, these effects should be easier to monitor by ³H n.m.r. if suitably ³H-labelled ligands are available.

The use of ³H n.m.r. to examine ³H-labelled bound ligands thus offers a valuable additional method of directly studying protein ligand complexes. Tritium has a relatively high natural sensitivity to n.m.r. detection (1.2 times greater than that of ¹H nuclei) and, because its chemical shifts are directly related to ¹H

chemical shifts, it is easy to compare the ^3H and ^1H results, the latter often being obtained from complex spectra where ambiguities in assignment can arise [16]. Notwithstanding the obvious potential of the technique, there are still relatively few examples of studies of protein–ligand interactions based on using ^3H -labelled ligands. In one study, Gehring and co-workers [17] used ^3H n.m.r. to monitor the anomeric binding specificity of α - and β -maltodextrins binding to a maltose-binding protein. In another investigation, O'Connell and co-workers [18] used ^3H n.m.r. to measure the dynamic properties of tosyl groups in specifically ^3H -labelled tosylchymotrypsin.

The present work examines the ^3H spectra of complexes of *L. casei* DHFR and selectively ^3H -labelled folates and MTX labelled at sites designed to probe multiconformational behaviour and dynamic processes in these complexes.

MATERIALS AND METHODS

DHFR was isolated from an *Escherichia coli* strain into which the *L. casei* DHFR gene had been cloned [19] and the enzyme was purified using methods described previously [20]. NADP^+ , folic acid, 3',5'-dibromofolic acid and MTX were obtained from Sigma. 5% Pd/CaCO₃ was obtained from Aldrich. 3',5'-Dichloro-MTX was prepared by published procedures [21]. The pH measurements were carried out using a Hanna Instruments H1 8417 pH-meter equipped with a combination glass electrode. The reported pH values are meter readings uncorrected for deuterium-isotope effects. In some cases, pH-dependent spectral features were used to calibrate the pH values measured in the two collaborating laboratories.

The samples of the DHFR · NADP^+ · folic acid complexes were prepared by adding solutions of the DHFR · NADP^+ binary complex to aliquots of the solid ^3H -labelled folic acids. The solution of the DHFR · NADP^+ binary complex was prepared by redissolving in 0.5 ml of $^2\text{H}_2\text{O}$, a freeze-dried enzyme sample containing 2 mM DHFR and 2 mM NADP^+ , 100 mM KCl, 50 mM potassium phosphate, adjusted to pH 6.5. The DHFR · MTX samples were prepared by adding 0.6 ml of a solution of DHFR (1.1 mM in 500 mM KCl/50 mM potassium phosphate in $^2\text{H}_2\text{O}$ at pH 6.5) to one equivalent of solid ^3H -labelled MTX. These procedures were adopted to minimize the handling of the ^3H -labelled ligands. (^3H is a weak β -emitter and all manipulations were carried out with the appropriate safety procedures for handling radioactive materials [16]. Samples were examined under double-containment conditions involving Teflon inserts in 5 mm n.m.r. tubes.)

The ^3H n.m.r. experiments were carried out on a Bruker AC-300 MHz spectrometer. Typically, 30000 transients were collected for spectra with widths of 3846 Hz and using 16000 data points. The ^3H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) by first measuring the shifts from the highest-field methyl ^1H resonance in the protein spectrum and then converting them into the DSS ^3H scale.

Preparation of [7,3',5'- $^3\text{H}_3$]folic acid

3',5'-Dibromofolic acid (5.0 mg, 0.078 mmol) and 5% Pd/CaCO₃ (10.2 mg) in 4% NaOH (0.5 ml) were stirred for 40 min in the presence of tritium gas (2 Ci) on the tritium gas line [22]. After this time, helium gas was allowed into the reaction vessel and the reaction mixture was stirred for a further 10 min to ensure the maximum incorporation of tritium gas. The catalyst was filtered out immediately through a cottonwool/Celite pad, and the filtrate was adjusted to pH 7.0 ± 1.0 with dilute HCl. The solution was then frozen in liquid nitrogen and freeze-dried. The crude folic acid was taken up in distilled water (0.5 ml), and any

insoluble material was solubilized with 4% NaOH; the solution was then chromatographed for 5 h on Whatman 3 MM chromatography paper using 0.5% K₂CO₃ solution as the developing solvent. The folic acid (R_f 0.6) was extracted with warm water and its concentration in solution determined spectrophotometrically ($\epsilon_{282} = 25100$ in 0.1 M NaOH) [23]. The solution was then freeze-dried. The average yield was about 50 mCi, and the estimated specific radioactivity at this stage was 40–50 Ci/mmol. The folic acid potassium salt was ion-exchanged on a DEAE-cellulose column (1 cm × 25 cm) to remove radiochemical impurities formed on storing the preparation [24]. The column was eluted with a linear gradient consisting of 250 ml water in the mixing flask and 250 ml of 2 M ammonium acetate, pH 6.9, in the reservoir. The u.v. absorption of the effluent was monitored at 288 nm with a Cecil CE 272 spectrophotometer [$\epsilon_{288} = 26000$ in water/2 M ammonium acetate, 1:1 (v/v)]. Fractions (7 ml) were collected with an automated sample collector. Eluted fractions were counted for radioactivity by pipetting 10 μl aliquots into 5 ml of Unisolve E scintillant. The fractions containing folic acid were pooled and freeze-dried until all the ammonium acetate was removed. The residue was dissolved in water and the folic acid content was determined spectrophotometrically. ^3H -labelled samples of folic acid were characterized by using ^3H n.m.r. and by comparing ^1H -n.m.r. spectra of the ^3H -labelled and non- ^3H -labelled samples. The average yield was 30 mCi of folic acid of specific radioactivity 32 Ci/mmol. The folic acid obtained was purged with nitrogen, sealed and stored at -18°C . The pattern of tritium labelling was determined using ^3H -n.m.r. spectroscopy (see Figure 1a): this indicated H-7 (32%); H-3',5' (64%); H-9 (4%). Thus most of the tritium is located in the 7,3',5' positions.

Preparation of [7,9- $^3\text{H}_2$]folic acid

Folic acid (10 mg, 0.023 mmol) and 5% Pd/CaCO₃ (37 mg) in 4% NaOH (750 μl) were stirred for 1.5 h in the presence of tritium gas (2 Ci) on the tritium gas line. Helium gas was then allowed into the reaction vessel and the reaction mixture stirred for a further 1 h to ensure maximum incorporation of tritium gas. The folic acid was then extracted from the solution by the procedure described above. After the Whatman-3MM-chromatography stage the average yield was about 60 mCi and the estimated specific radioactivity was 10–20 Ci/mmol. The folic acid was further purified as described above and the average yield was 15–20 mCi of folic acid of specific radioactivity 5 Ci/mmol. The folic acid sample was purged with nitrogen, sealed and stored at -18°C . The pattern of tritium labelling was determined by ^3H n.m.r.; this indicated H-7 (40%); H-9 (57%); H-3',5' (3%) (see Figure 1b). Thus most of the tritium is located in the 7,9 positions.

Preparation of [7,3',5'- $^3\text{H}_3$]MTX

3',5'-Dichloro-MTX (6.5 mg, 0.012 mmol) and 5% Pd/CaCO₃ (24.3 mg) in 4% NaOH (0.75 ml) were stirred for 16 h in the presence of tritium gas (2 Ci) on the gas line [25]. Helium gas was then allowed into the reaction vessel and the reaction mixture stirred for a further 1 h to ensure maximum incorporation of tritium gas. The MTX was then extracted from the solution by the procedure described above. After the Whatman-3 MM-chromatography stage, the average yield was about 40 mCi and estimated specific radioactivity was 8–10 Ci/mmol. The MTX was further purified and characterized as described above for folic acid, and the average yield was 5–10 mCi of MTX of specific radioactivity 9 Ci/mmol. The MTX obtained was purged with N₂, sealed and stored at -18°C . The pattern of ^3H labelling

Figure 1 ^3H -n.m.r. spectra at 320 MHz of (a) $[7,3',5'\text{-}^3\text{H}_3]$ folic acid (pH 7.6, 19.1 mM), (b) $[7,9\text{-}^3\text{H}_3]$ folic acid (pH 7.8, 8.4 mM) and (c) $[7,3',5'\text{-}^3\text{H}_3]$ MTX (pH 7.4, 0.83 mM)

was determined by ^3H n.m.r.; this indicated H-7 (57%); H-3',5' (43%) (see Figure 1c).

RESULTS AND DISCUSSION

$[7,9\text{-}^3\text{H}_3]$ - and $[7,3',5'\text{-}^3\text{H}_3]$ -folic acids and $[7,3',5'\text{-}^3\text{H}_3]$ MTX

^3H n.m.r. spectra of the free ^3H -labelled folic acids and MTX are shown in Figure 1. The sites of tritiation can be deduced by comparing the observed signals with the assigned ^1H chemical shifts of free non-labelled folic acid and MTX. The relative levels of tritiation were measured by integrating the ^3H signals in the spectra.

DHFR·NADP⁺· $[7,9\text{-}^3\text{H}_3]$ -folic acid complex

This complex was examined at a series of pH values in the presence of 2 equiv. of $[7,9\text{-}^3\text{H}_3]$ folic acid, and the ^3H spectra are shown in Figure 2. One equivalent of folate binds tightly to DHFR and the second equivalent remains free in solution, the bound and free species being in slow exchange. At pH 7.4 (Figure 2a), two signals were observed for bound ^3H -7 (8.00 and 9.45 p.p.m) corresponding in chemical shift to the H-7 signals of the two conformations IIa and IIb observed previously at high pH in ^1H two-dimensional exchange experiments [26]. The ^3H -7 signals of both the bound and free folate are broad (~ 40 Hz), and this reflects exchange between the two species. When the pH is decreased, a new signal appears in the ^3H -7 region and this corresponds to the H-7 proton signals detected previously (8.30 p.p.m.) for Form I of the complex. It can be seen in Figure 2(d) that ^3H -7 signals for all three forms are present at pH 5.3.

Signals for the bound ^3H -9 were also detected in the spectra. At pH 7.4 (Figure 2a) these signals appeared at 4.15, 3.85 and 3.15 p.p.m. The observed signals are even broader than the ^3H -7 signals; part of the line broadening is from ^1H - ^3H spin coupling (estimated to be ~ 20 Hz), but there could be a further

Figure 2 ^3H -n.m.r. spectra at 320 MHz of $[7,9\text{-}^3\text{H}_3]$ folic acid in a 1:1:1 complex with *L. casei* DHFR and NADP⁺ at different pH values in the presence of an additional equivalent of free folic acid

(a) pH 7.4, 25 °C; (b) pH 6.6, 25 °C; (c) pH 6.0, 25 °C; (d) pH 5.3, 25 °C; (e) pH 7.4, 10 °C. Each spectrum was obtained from a 15 h accumulation (2 mM DHFR, 4 mM $[7,9\text{-}^3\text{H}_3]$ folic acid, 100 mM KCl, 50 mM potassium phosphate in $^2\text{H}_2\text{O}$). Samples examined a few days after preparation gave spectra containing some small additional signals from breakdown products: these are marked 'x' (at 8.6 and 9.3 p.p.m.) in (d).

contribution to the linewidth from overlap of signals with different chemical shifts. The two protons (or tritons) at position 9 will be non-equivalent in bound folate, and thus four signals for the ^3H -9 nuclei can be anticipated, corresponding to the two different conformations. Johnson and Bovey [27] ring-current-shift calculations based on the crystal structure data of Bolin et al. [2] (for the *L. casei* DHFR·MTX·NADPH complex) predict that one of the two protons (or tritons) on C-9 has an upfield shielding contribution of 0.9 p.p.m. This would correspond closely to the signal observed at 3.15 p.p.m.

The ^3H -9 signals for bound folate are also seen at pH 5.3 (at 3.95 and 3.15 p.p.m.) (Figure 2d), where all three forms are present. The similarity in the shifts at pH 7.4 and 5.3 indicates that the ^3H -9 nuclei are in similar environments in Forms I, IIa and IIb. The sample used to obtain Figure 2(d) had been prepared for 5 days, and its spectrum indicates that there is a small amount of folate decomposition. This gives rise to small signals at 8.6 and 9.3 p.p.m. (marked 'x' on Figure 2d). These

Figure 3 ^3H -n.m.r. spectrum at 320 MHz of $[7,3',5'\text{-}^3\text{H}_3]$ folic acid in a 1:1:1 complex with *Lcase*/ DHFR and NADP^+ at pH 7.4 and 25 °C

The spectrum was obtained from a 15 h accumulation. Concentrations: DHFR, 2 mM; $[7,3',5'\text{-}^3\text{H}_3]$ folic acid, 2 mM.

Figure 4 ^3H -n.m.r. spectrum at 320 MHz of $[7,3',5'\text{-}^3\text{H}_3]$ MTX (a) alone, (b) in a 1:1 complex with *Lcase*/ DHFR (1.1 mM) at pH 6.5, 283 K and (c) in a 1:1:1 complex with *Lcase*/ DHFR and NADPH at pH 6.5 and 313 K

Spectra in (b) and (c) were obtained from 15 h accumulations.

signals increase in intensity in spectra of samples examined 14 days later (spectra not shown).

As a final experiment, the pH of the sample was re-adjusted from 5.3 to 7.4 and the ^3H spectrum recorded (Figure 2e); this showed the same intensities for the signals from the different conformational forms as seen in the previously obtained spectra recorded at pH 7.4. This indicates that the pH-induced inter-conversion between the conformational states is reversible. The spectrum in Figure 2(e) was recorded at a lower temperature (283 K) in order to examine the effects of exchange; the observed

Figure 5 Calculated spectra for various exchange rates between two equally populated non-equivalent sites in exchange with each other

lines are narrower, as would be expected from a decrease in exchange rate between bound and free species.

Previous measurements on the DHFR· NADP^+ ·folic acid complex using non- ^3H -labelled ligands indicated that the multiple conformations are present, with the same pH dependence, in solutions with different ionic strengths (100–500 mM KCl) [13,14,28].

DHFR· NADP^+ · $[7,3',5'\text{-}^3\text{H}_3]$ folic acid complex

The ^3H -n.m.r. spectrum of this complex recorded at pH 7.4 is shown in Figure 3. Again the two distinct ^3H -7 bound signals were detected confirming the existence of the two conformations present at high pH in this complex (Forms IIa and IIb). By contrast, only a single resonance was detected for the $3',5'\text{-}^3\text{H}$ nuclei from the benzoyl ring of bound folate (5.60 p.p.m.), and there is a substantial chemical-shift difference between the bound and free species (the signal from bound is 1.26 p.p.m. upfield of the free). The chemical shift of the $3',5'\text{-}^3\text{H}$ nuclei in bound folate at pH 5.3 (5.60 p.p.m.) is the same as seen at pH 7.4 (spectrum not shown). Thus the appearance of a single resonance, well shifted from the position of the free, indicates that the benzoyl ring is binding in essentially the same binding site in all three conformations. Furthermore, the equivalence of the 3' and 5' chemical shifts shows that this ring is undergoing relatively rapid ring flipping.

DHFR· $[7,3',5'\text{-}^3\text{H}_3]$ MTX complex and ternary complex with NADPH

The ^3H -n.m.r. spectrum of free MTX is shown in Figure 4(a). A comparison with the spectrum of the binary complex with MTX

is shown in Figure 4(b) and indicates that the only bound ligand signal observed is ³H-7, no signal being seen for the 3',5' nuclei. Similar results were obtained for the ternary complex with NADPH (Figure 4c). Varying the sample temperature over the range 283–313 K did not noticeably change the ³H spectra of the binary or ternary complex. The most obvious explanation for the absence of the 3',5' signal(s) is that they have been broadened by some exchange process such as, for example, flipping of the benzoyl ring. This is consistent with our previous ¹H-n.m.r. studies of MTX complexes with DHFR, where signals for the 3',5' protons have never been detected for either the binary or ternary complexes. Ring-current chemical-shift calculations (Johnson and Bovey [27]) based on the crystal structure data of Bolin and co-workers [2] for the DHFR·MTX·NADPH complex estimate that the difference in chemical shifts of the 3' and 5' protons is 1.36 p.p.m. (435 Hz at 320 MHz). We have used McConnell's modified Bloch equations [29] to calculate the spectra for signals corresponding to exchange at various rates between two equally populated non-equivalent sites having a chemical shift difference of 435 Hz. A noise level corresponding to that observed in the experimental spectrum was included in the simulation. Figure 5 shows the spectra calculated for various rates over the range 250 s⁻¹ to 10⁶ s⁻¹; it is seen that, for rates between 500 to 4000 s⁻¹, the signals are so broad that they would not have been detected at the prevailing signal-to-noise ratio. The rates of flipping of the benzoyl ring are not known for these complexes, but they have been measured for binary and ternary complexes of DHFR with 3',5'-difluoro-MTX and NADPH [15], where values of 7000 s⁻¹ at 298 K (binary) and 20000 s⁻¹ at 298 K (ternary) were obtained. From the activation energies determined in that study, one can estimate that an eightfold increase in rate (from 500 to 4000 s⁻¹) would be achieved by increasing the temperature by 30 °C (from 283 to 313 K). Thus the observed rates of ring flipping over the temperature range used to examine the DHFR complexes with the ³H-labelled MTX are somewhat lower than, but not too dissimilar from, those measured previously for the complexes with 3',5'-difluoro-MTX.

Conclusions

These findings indicate that ³H n.m.r. provides a very direct method for examining multiconformational states and dynamic processes in protein–ligand complexes. The presence of the three pH-dependent forms of bound folate has been confirmed.

The major differences in the conformation of the pterin ring in the different forms have been characterized previously. The present results, by providing information on the similar chemical shifts of the tritons on C-9 and C-3' and C-5' of the benzoyl ring in bound folate, indicate that the non-pterin part of folate is binding in a similar way in the different forms. Ring flipping rates for the benzoyl ring in bound folate are sufficiently high to give an averaged signal for the 3',5' tritons.

The results for the complexes of DHFR with MTX and NADPH indicate that a single major conformation exists for the binary DHFR·MTX complex and also for the ternary complex with NADPH. The line-broadening which prevents observation

of a signal for the 3',5' tritons of the benzoyl ring in bound MTX is consistent with the ring flipping being insufficiently fast to give chemical shift averaging of the 3',5' signals.

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