^{13}C and ^{15}N Nuclear Magnetic Resonance Evidence of the Ionization State of Substrates Bound to Bovine Dihydrofolate Reductase†

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ABSTRACT: The state of protonation of substrates bound to mammalian dihydrofolate reductase (DHFR) has significance for the mechanism of catalysis. To investigate this, dihydrofolate and dihydropteroylpentaglutamate have been synthesized with ^{15}N enrichment at N-5. ^{15}N NMR studies have been performed on the binary complexes formed by bovine DHFR with these compounds and with [5-^{15}N]dihydrobiopterin. The results indicate that there is no protonation at N-5 in the binary complexes, and this was confirmed by ^{13}C NMR studies with folate and dihydrofolate synthesized with ^{13}C enrichment at C-6. The chemical shift displacements produced by complex formation are in the same direction as those which result from deprotonation of the N-3/C-4-O "amide" group and are consistent with at least partial loss of the proton from N-3. This would be possible if, as crystallographic data indicate, there is interaction of N-3 and the 2-amino group of the bound ligands with the carboxylate of the active site glutamate residue (Glu^{30}).

The enzyme mechanism of dihydrofolate reductase (NADP:5,6,7,8-tetrahydrofolate oxidoreductase, EC 1.5.1.3, DHFR)[1] is of interest in part because some inhibitors like methotrexate are anticancer agents, others like pyrimethamine are antimalarials, and others like trimethoprim are antibacterial agents. X-ray crystallography has revealed the positioning of such inhibitors in the catalytic site and indicated that they make an ionic, hydrogen-bond interaction with the active site carboxyl [for reviews, see Blakley (1984) and Freisheim and Matthews (1984)]. This interaction was confirmed by NMR (Cocco et al., 1981a,b, 1983; Roberts et al., 1981). However, indirect evidence indicates that H_2folate and related substrates bind with their pteridine ring rotated 180° compared with MTX. This has recently been confirmed by X-ray crystallographic data for the complex of folate with human DHFR (Oefner et al., 1988). As anticipated, these data also show that folate makes a very different interaction with the active site carboxyl from that made by MTX.

When X-ray crystallography revealed that a carboxyl is the only ionizable group in the active site of DHFR from both bacterial and vertebrate sources, it was proposed that this group is at least partially in its protonated form at neutral pH and acts as a proton donor to the substrate, thus assisting acceptance of a hydride ion from NADPH (Matthews et al., 1978). Evidence to support this view was obtained from a study of mutants of DHFR from *Escherichia coli* (ECDHFR) in which the active site aspartate (Asp^{27}) is replaced by asparagine or serine (Howell et al., 1986). However, direct protonation of H_2folate by the active site carboxyl (Asp^{27} in ECDHFR, Glu^{30} in eukaryotic DHFRs) is not possible since it hydrogen bonds with N-3 and the 2-amino group of the substrate (Oefner et al., 1988). A modified scheme involving intermediate donation of a proton from the active site carboxyl to a bound water, subsequent formation of the enol tautomer of bound folate or H_2folate, and proton transfer from the enol to N-5 has been proposed (Taira et al., 1987). Ab initio calculations have been made predicting that formation of the enol tautomer with hydrogen bonding of the enolic group to N-5 would increase the negative charge on N-5 and the positive charge on C-6 (Uchimaru et al., 1989).

In order to explore the state of protonation of substrates bound to DHFR by the use of NMR, we have synthesized substrates labeled with ^{15}N at N-5 or ^{13}C at C-6 and, because of the clinical relevance of the mammalian enzyme, have chosen to work with bovine DHFR (BDHFR).

Materials and Methods

Materials. H_2folate and labeled forms of it were prepared from folate and labeled folate (Blakley, 1960). H_2biopterin and [5-^{15}N]H_2biopterin were obtained from Dr. B. Schircks laboratory (Jona, Switzerland). Ampholines for isoelectric focusing were obtained from Pharmacia LKB (Bromma, Sweden). BDHFR was prepared according to Kaufman and Kemmerer (1977), except that for some preparations enzyme was eluted from the methotrexate–Sepharose, packed in a

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[1] Abbreviations: DHFR, dihydrofolate reductase; NMR, nuclear magnetic resonance; ECDHFR, dihydrofolate reductase from *Escherichia coli*; MTX, methotrexate; H_2folate, 7,8-dihydrofolate; BDHFR, bovine dihydrofolate reductase; TLC, thin-layer chromatography; UV, ultraviolet; $H_2PteGlu_5$, 7,8-dihydropteroylpentaglutamate; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetate; DMSO, dimethyl sulfoxide; H_2biopterin, 7,8-dihydrobiopterin.

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100-mL radial column (Sepragen, San Leandro, CA), by folate instead of by H_2folate as in the original method. Folate or H_2folate was removed in three steps: gel filtration on Sephadex G-100, treatment of the preparation with Amberlite CG-400 (Sigma) after concentration by ultrafiltration, and isoelectric focusing for 24 h. Initially, an Ampholine with a pH range of 3.5–10 was used, but better results were obtained with a pH range of 5–8. Specific activity of preparations was about 60 IU/mg. Preparations were stable when frozen at −70 °C. In preparation for NMR experiments the concentrated enzyme was diluted with an equal volume of 1.0 M KCl in 0.1 M potassium phosphate buffer, pH 7.3, containing 4 mM EDTA and reconcentrated by ultrafiltration. The concentrate was diluted with 3 volumes of 0.5 M KCl containing 50 mM potassium phsphate buffer, pH 7.3, 2 mM EDTA, and 20% D_2O and again concentrated to 1.0–1.5 mM. The final concentration was determined by titration of enzyme fluorescence with methotrexate as previously described (Williams et al., 1979). There was negligible loss of enzyme activity during collection of NMR transients for 2 days at 10 °C.

[5-^{15}N]Folic Acid. 5-[^{15}N]Nitroso-2,4,6-triaminopyrimidine was prepared from $Na^{15}NO_2$ (Merck, Sharp and Dohme Isotopes) and 2,4,6-triaminopyrimidine (Aldrich), which were reacted according to Konrad and Pfleiderer (1970). The yield was close to theoretical on the basis of both the pyrimidine and nitrite. [5-^{15}N]-2,4,6-Diamino-6-(hydroxymethyl)pteridine was synthesized from the 5-[^{15}N]nitroso-2,4,6-triaminopyrimidine according to Boyle and Pfleiderer (1980). From 6.67 g of the pyrimidine the yield of pteridine was 5.86 g (59%). This material appeared by TLC on cellulose in butanol–acetic acid–water (4:1:5, upper layer) to have negligible contaminating impurities. The (hydroxymethyl)pteridine was converted to [5-^{15}N]-2,4-diamino-6-(bromomethyl)pteridine hydrobromide according to the procedure of Piper and Montgomery (1977) but without the intermediate formation of the hydrobromide. A yield of 1.56 g (15%) of crystalline material was obtained, and further amounts of noncrystallizable material were obtained from the benzene extracts. The latter was purified by solution in 0.1 N HCl and precipitation by adjustment of the pH to 4. After being washed with cold water and dried in vacuum (20 μgHg) over P_2O_5 this material (1.48 g, 14.5%) appeared by TLC, performed as previously described, to be almost as pure as the crystalline material. The latter had a small amount of the 6-methyl derivative as contaminant. Conversion to [5-^{15}N]aminopterin by reaction with (*p*-aminobenzoyl)glutamic acid in dimethylacetamide was performed according to Piper and Montgomery (1977). The yield from the crystalline bromomethyl compound was 1.7 g (83%). TLC on cellulose in 50 mM potassium phosphate buffer, pH 7.4, showed a single UV-absorbing spot with the same R_f as authentic aminopterin. The product from the uncrystallized [5-^{15}N]diamino(bromomethyl)pteridine was dark in color but appeared to be relatively pure by TLC. It was purified first by solution at pH 8, filtration through Celite, and reprecipitation at pH 3.6 and then by chromatography on a DEAE-cellulose (DE52 Whatman) column (3.4 × 34 cm) with elution with a gradient obtained with 1 L of 0.02 M ammonium bicarbonate in the mixer and 1 L of 2.0 M ammonium bicarbonate in the reservoir. Elution was continued with 2.0 M ammonium bicarbonate until the [5-^{15}N]aminopterin peak had been completely eluted. Fractions (15 mL) were monitored at 260 nm. The fractions in the aminopterin peak were pooled and evaporated to dryness on a rotary evaporator. The residue was dissolved in a minimum volume of water and added dropwise to 800 mL of 1 N acetic acid at 80 °C. pH was adjusted to 3.5 and aminopterin separated in a granular form during cooling to room temperature and overnight at 5 °C. The yield of chromatographically pure material was 0.39 g. [5-^{15}N]Folic acid was obtained by dissolving 1.0 g of [5-^{15}N]aminopterin in 70 mL of 1 N NaOH that had been degassed with water-saturated helium for 2.5 h. The solution was heated at 100 °C for 5 h with protection from light while a slow stream of water-saturated helium continued to pass through the solution. The solution was cooled, adjusted to pH 7.4, and added dropwise with stirring to 800 mL of 1 N acetic acid at 80 °C. The granular precipitate thus obtained was dissolved in dilute ammonia, adjusted to pH 7–8, and applied to a DE52 column for chromatographic purification, as in the case of aminopterin. Yield of [5-^{15}N]folic acid was 807 mg (74% assuming the dihydrate).

[6-^{13}C]Folic Acid. 2,4,5,6-Tetraaminopyrimidine was prepared from the triaminopyrimidine according to the method of Boyle and Pfleiderer (1980). When the filtered solution of the tetraaminopyrimidine was reacted with commercial dihydroxyacetone according to the method of these authors, the yield of 2,4-diamino-6-(hydroxymethyl)pteridine was 24% on the basis of dihydroxyacetate. This could be increased to 35% by decreasing the molar ratio of dihydroxyacetone to tetraaminopyrimidine to 1.57.

For the synthesis of [2-^{13}C]dihydroxyacetone we initially attempted the synthetic route used by Gatenbeck (1955) for [2-^{14}C]dihydroxyacetone, but yields of the product in a satisfactory state of purity were low. A better route was based on [2-^{13}C]-D-glucose, which was prepared by the efficient route described by Barker and co-workers (Hayes et al., 1982). This starting material was isomerized by a commercial preparation of glucose isomerase to [2-^{13}C]-D-fructose (Walker et al., 1988), which was converted to a mixture of its methyl glycosides and treated with sodium metaperiodate. The resulting dialdol compounds were reduced with sodium borohydride and hydrolyzed to yield a mixture of [2-^{13}C]dihydroxyacetone, glycerol, and ethylene glycol. A concentrate of this mixture of product was used directly in the synthesis.

Condensation of [2-^{13}C]dihydroxyacetone in this preparation with the tetraaminopyrimidine proceeded much more slowly than in the case of commercial unlabeled dihydroxyacetone. Whereas the reaction of the unlabeled material was complete in 18 h at 5 °C, the labeled material required several weeks at room temperature. Because of the slowness of the condensation, there was no necessity to bubble oxygen through the solution to oxidize the dihydropteridine intermediate. In order to prevent degradation of the tetraaminopyrimidine over the prolonged reaction period, additional amounts of cysteine hydrochloride (each equal to that originally used by Boyle and Pfleiderer) were added at intervals of 3 days. After each addition the pH of the mixture was adjusted to 5 with ammonia. The precipitate that formed during the reaction period contained some cysteine which was removed by solution at pH 1, filtration, and reprecipitation by adjustment of the filtrate to pH 5 with ammonia. After being allowed to stand overnight at 0 °C, the precipitate was filtered off, washed with cold water, and dried under vacuum (20 μmHg) over P_2O_5 at room temperature. TLC on cellulose in butanol–acetic acid–water showed a major blue fluorescing spot (R_f 0.48) and a minor blue fluorescing faster spot. A total of 3.16 g was obtained from 14.1 g of the syrup containing [2-^{13}C]dihydroxyacetone, glycerol, and ethylene glycol.

Conversion of this material to [6-^{13}C]-2,4-diamino-6-(bromomethyl)pteridine hydrobromide was performed as in the case of the ^{15}N-labeled material, but the product could not

be crystallized and was instead purified by extraction first with acetic acid and, after removal of solvent, with 2-propanol. After condensation with (*p*-aminobenzoyl)glutamate, the product was isolated as in the procedure of Piper and Montgomery (1977), dissolved in dilute ammonia, and purified on DE52 as described for [5-^{15}N]aminopterin. The yield was 612 mg of chromatographically pure [6-^{13}C]aminopterin. A portion of this (503 mg) was converted to [6-^{13}C]folic acid (352 mg, 65% assuming the dihydrate), by the same procedure used for the ^{15}N compounds.

[5-^{15}N]PteGlu$_5$ and [6-^{13}C]PteGlu$_5$. Some of the labeled folate that had been synthesized was converted to labeled pteroic acid by a slight modification of the procedure previously used (Peterson et al., 1975). Yields were 218 mg (84%) for [5-^{15}N]pteroic acid and 131 mg (97%) for [6-^{13}C]pteroic acid. The labeled pteroic acids were converted to N^2,N^{10}-bis(trifluoroacetyl)pteroic acid according to Krumdieck and Baugh (1980). Yields were as follows: ^{15}N labeled, 241 mg (71%); ^{13}C labeled, 153 mg (75%). Synthesis of resin-bound protected pentaglutamic acid and coupling to protected pteroic acid were also carried out according to Krumdieck and Baugh (1980). The Merrifield resin used was from Sigma (1% cross-linked, 1.3 mequiv Cl/g). Cleavage, deprotection, and purification were performed as described except that elution from the ion-exchange column was with a gradient of NaCl in 10 mM glycylglycine buffer, pH 7.5. The yield was 42% on the basis of the protected pteroic acid. The products had correct ultraviolet spectra in 0.1 N NaOH with regard to wavelengths and ratios of absorbance maxima. Solutions were concentrated by ultrafiltration on YCO5 membranes (Amicon). Analytical HPLC was performed on a C_{18} Altex ODS column (4.6 × 250 mm) with a flow rate of 0.9 mL/min and detection at 254 nm. The column was initially equilibrated with solvent A (5 mM tetrabutylammonium phosphate, pH 7.5, in 42% methanol). Two minutes after sample injection, a linear gradient was commenced to 100% solvent B over 38 min (solvent B, 5 mM tetrabutylammonium phosphate, pH 7.5, in 60% methanol). The major peak (82%) eluted at 36 min. Smaller peaks (33 min, 4.5%; 39 min, 12%) probably represent the tetra- and hexaglutamate.

H_2folate and H_2PteGlu$_5$. H_2folate was prepared as previously (Blakley, 1960), and the microcrystalline solid was stored as a suspension in 1 mM HCl containing 100 mM 2-mercaptoethanol at −70 °C. Reduction of the pentaglutamate was carried out similarly. To labeled PteGlu$_5$ (20 μmol) in 3.6 mL, 0.4 g of sodium ascorbate was added and dissolved with magnetic stirring. The pH was adjusted to 6.0 with acetic acid, and 0.16 g of sodium dithionite was added with stirring. After 5 min the solution was added to a Sephadex G-10 column (1.1 × 60 cm) that had been equilibrated at 5 °C with 50 mM Tris-HCl, pH 7.3, containing 5 mM dithiothreitol (Sigma) and 0.02% sodium azide. Elution was performed at 5 °C with the same buffer, and fractions of 2.2 mL were collected. Most of the material absorbing at 280 nm appeared in a single peak with the maximum at fractions 13–16 in different preparations. Two or three of the peak fractions were combined, a sample was removed for determination of the absorbance spectrum and enzymic estimation of the purity, and the remainder was quickly frozen and stored at −70 °C. The concentration of the combined fractions was 1.5–2.5 mM, and the purity was 93% by enzymatic assay (total absorbance change at 340 nm in the presence of DHFR and a molar excess of NADPH).

Dissociation Constants. These were determined by titration of protein fluorescence as previously described (Appleman et al., 1988). The enzyme solution contained the same constituents as in NMR experiments, and the temperature was 10 °C.

Kinetic Constants. These were determined spectrophotometrically under usual assay conditions for BDHFR: 0.5 M sodium acetate buffer, pH 6.0, containing 0.6 M KCl. The concentration of NADPH was 100 μM.

NMR Methods. NMR spectra were obtained on a General Electric GN-500 NMR spectrometer operated with quadrature detection and deuterium lock. The probe temperature was maintained at 10 °C for all experiments unless otherwise stated. The solution pH was adjusted by addition of NaOD and DCl solutions and measured with a glass electrode.

A frequency of 50.68 MHz was utilized for ^{15}N spectra, and chemical shifts are reported relative to the center of the ^{15}N multiplet of formamide contained in a capillary. ^{13}C NMR spectra were acquired at 125.76 MHz with the cyclooctane resonance at 26.3 ppm (downfield from TMS) in external CH_2I_2/cyclooctane as a reference standard. Flip angles of 30° for ^{15}N and 45° for ^{13}C were used. Other conditions used for acquisition of spectra with enzyme present are shown in the figure legends. Similar conditions were used for the titrations, but fewer transients were necessary (ca. 2000).

For titration of uncomplexed ligands, 1.5–2-mL solutions in 50 mM buffer with 10–20% D_2O added were utilized. In the case of H_2folate and its pentaglutamate analogue, 0.1 M 2-mercaptoethanol was present as well. The buffer utilized was 50 mM potassium phosphate, except for [5-^{15}N]H_2folate (50 mM Tris containing 0.43 M mercaptoethanol and 2% EDTA) and the pteroylpentaglutamate (1–5 mM glycylglycine). Spectra of [5-^{15}N]H_2biopterin in buffer and [6-^{13}C]folate in 50% DMSO were acquired at 25 °C, and the chemical shifts given in Figure 1 for these compounds are corrected to 10 °C on the basis of comparison with shifts obtained at this temperature.

For spectra in the presence of enzyme, solutions of 10–20 mM ligand were added to solutions of DHFR (ca. 1 mM) in 50 mM potassium phosphate buffer (0.5 M KCl, 2 mM EDTA, and 20% D_2O at pH 7.3) to give the desired ratios of ligand to enzyme in 1.5 mL. A solution of H_2folate was prepared by neutralizing a microcrystalline suspension in 1 mM HCl with 1.0 M dipotassium hydrogen phosphate and added to the enzyme solution. All enzyme solutions were maintained at 10 °C.

Results

Effect of pH on Shifts of Labeled Substrates. Although there have been a number of reports, summarized in a recent review (Temple & Montgomery, 1984), of the chemical shifts of folate and reduced derivatives, there has been little systematic analysis of the effects of pH on spectral parameters. Such data are difficult to obtain as a consequence of the limited solubility of many of these derivatives at low pH, a significant degree of self-association at higher concentrations (Poe, 1973), and, in the case of reduced derivatives, limited stability. The results of studies of pH dependence of shift for [5-^{15}N]folate and derivatives, for [5-^{15}N]H_2biopterin, and for [6-^{13}C]folate and derivatives are shown in Figure 1.

Below pH 6, the ^{13}C or ^{15}N resonances of folate and H_2-folate undergo a marked displacement and dramatic broadening reflecting aggregation and precipitation so that it was not possible to get meaningful shifts below this pH. ^{13}C NMR for folate in DMSO–D_2O yielded a pH titration curve with pK values (2.58 and 8.56) in reasonable agreement with literature values for folate (Poe, 1977). However, the titration shift difference for folate between high and neutral pH cor-

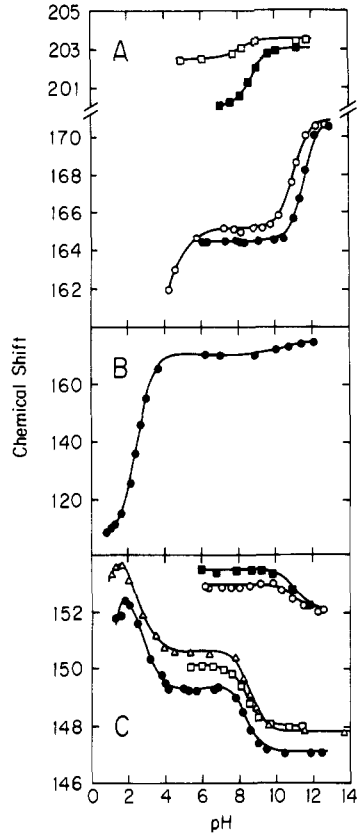

FIGURE 1: Dependence of chemical shift (in ppm) of labeled pteridines at 10 °C on pH. (A) (●) [5-^{15}N]H_2folate (3 mM); (○) [5-^{15}N]H_2PteGlu$_5$ (2 mM); (■) [5-^{15}N]folate (15 mM); (□) [5-^{15}N]-PteGlu$_5$ (3.5 mM). (B) 5-^{15}N]H_2biopterin (5 mM). (C) (○) (6-^{13}C]H_2folate (2 mM); (■) [6-^{13}C]H_2PteGlu$_5$ (1.4 mM); (□) [6-^{13}C]folate (1 mM); (▲) [6-^{13}C]folate in 50% DMSO–40% D_2O–10% buffer (0.5 mM); (●) [6-^{13}C]PteGlu$_5$ (3 mM).

responding to deprotonation of the N-3/C-4–O group was slightly larger in DMSO/D_2O than in H_2O. ^{13}C NMR studies of [6-^{13}C]pteroylpentaglutamate yielded a pH titration curve parallel to those for folate (Figure 1C). The shift differences between the mono- and pentaglutamate may in part reflect greater aggregation by the monoglutamate.

^{15}N NMR studies of [5-^{15}N]folate, [5-^{15}N]H_2folate, and their pentaglutamate analogues yield analogous results (Figure 1A). H_2biopterin is soluble and stable enough for shift measurements over much of the pH range, and it was possible to obtain a pK_a for protonation of both the amide group and N-5 (Figure 1B). The pK_a values for the latter compound (2.53 ± 0.01 and 10.43 ± 0.08) are in excellent agreement with those obtained spectrophotometrically and by proton NMR titration (Maharaj et al., unpublished results).

Shift of Bound [5-^{15}N]Dihydrobiopterin. Dihydrobiopterin is a substrate for DHFR, though in the case of BDHFR the apparent K_m is somewhat higher and k_{cat} considerably lower than for H_2folate (Table I). The dissociation constant, K_D, for the binary complex is also considerably higher. It is, however, reasonable to assume that it binds in the catalytic site in a similar way to dihydrofolate, although the disposition of the side chain may be different. Unpublished crystallographic evidence indicates that this is the case for H_2biopterin (Freisheim & Matthews, 1984). For NMR studies [5-^{15}N]-dihydrobiopterin was mixed with a 43% molar excess of BDHFR, with a calculated binding of 92% of the ligand. Only one resonance was observed, with a chemical shift of 174.47 ppm. With higher ratios of dihydrobiopterin to BDHFR (1.3:1 and 2:1), two resonances appeared in the spectrum, one corresponding to the bound ligand (174.39 ppm) and the other (171.18 ppm) corresponding to unbound ligand. The chemical shift of the latter corresponds to that shown in Figure 1B for free H_2biopterin at neutral pH. It is clear that there is no indication of more than one mode of binding of the labeled ligand and that there must be slow exchange between bound and unbound ligand. It is also clear that there is no protonation of the bound ligand, since the upfield shift upon protonation (Figure 1B, upper panel) is so large (≈60 ppm) that even partial sharing of a proton by N-5 would have produced a measurable upfield displacement of the shift. In reality, the change in chemical shift on binding to the enzyme more closely resembles that occurring when unbound H_2biopterin ionizes the amide (N-3/C-4–O) proton at high pH (Figure 1B).

Table I: Binary Dissociation Constants and Kinetic Constants for Ligands with BDHFR

ligand	K_D (μM)[a]	$K_{m,app}$ (μM)[b]	k_{cat} (s^{-1})[b,c]
folate	1.69 ± 0.10	4.28 ± 0.31[d]	0.15 ± 0.003[d]
H_2folate	1.58 ± 0.15	1.93 ± 0.19	8.9 ± 0.3
H_2PteGlu$_5$		2.50 ± 0.26	5.8 ± 0.2
H_2biopterin	31.8 ± 3.5	7.21 ± 1.1	0.69 ± 0.05

[a] From titration of protein fluorescence. [b] At 100 μM NADPH, except as indicated. [c] $k_{cat} = V_{max}$/(enzyme concentration). [d] At 200 μM NADPH.

Shift of Bound [5-^{15}N]H_2folate and [6-^{13}C]H_2folate. The variation of chemical shift with pH for [5-^{15}N]H_2folate could be determined only over a more limited range than for [5-^{15}N]H_2biopterin. However, it is to be expected that the effect of pH on the shift of ^{15}N in the two compounds will be very similar; i.e., there will be a large upfield change on protonation of N-5 and a small downfield movement on deprotonating N-3/C-4–O. Spectra A and B of Figure 2 are the ^{15}N NMR spectra obtained from [5-^{15}N]H_2folate in the presence of BDHFR when the ligand to enzyme ratio is 0.7:1 and 2.1:1. At the lower ratio a single resonance was observed with a chemical shift of 167.5 ppm as compared with 167.2 ppm for the downfield peak in Figure 2B. The upfield peak in Figure 2B has a shift of 164.8 ppm, which agrees with a shift of 164.5 ppm for the compound measured in the absence of enzyme at the same pH. Thus, binding to the enzyme causes a downfield shift for the ^{15}N resonance (2.4 ppm) similar to that observed for H_2biopterin (3.2 ppm).

The spectrum of [6-^{13}C]H_2folate in excess over BDHFR (Figure 2D) shows that binding to BDHFR results in a significant upfield shift, and it is again apparent that only a single conformation of the complex is present and that the shift of bound ligand is in slow exchange with that of free ligand. The upfield resonance in Figure 2D agrees well with that of the single resonance seen when enzyme is in molar excess (149.80 ppm), and the shift of the downfield peak agrees with that for unbound ligand at the same pH (Figure 1C). It should be noted that the effect on the NMR spectrum of binding the ligand to enzyme is in the same direction as, but significantly greater than (2.4 versus 0.8 ppm), the effect of deprotonating the amide group and is in the opposite direction to that of protonation of N-5.

Shift of Bound [5-^{15}N]H_2PteGlu$_5$. The polyglutamates were synthesized because they have greater solubility at low pH and are less subject to self-association, two of the problems that limit determination of chemical shifts for H_2folate at low pH. NMR spectra for the polyglutamates could be obtained for pH values as low as 4, and these data confirmed the direction in which the chemical shift is displaced as N-5 is protonated. Enzyme-bound [5-^{15}N]H_2PteGlu$_5$ had a chemical shift of 167.2 ppm compared with 165.2 ppm for the unbound ligand

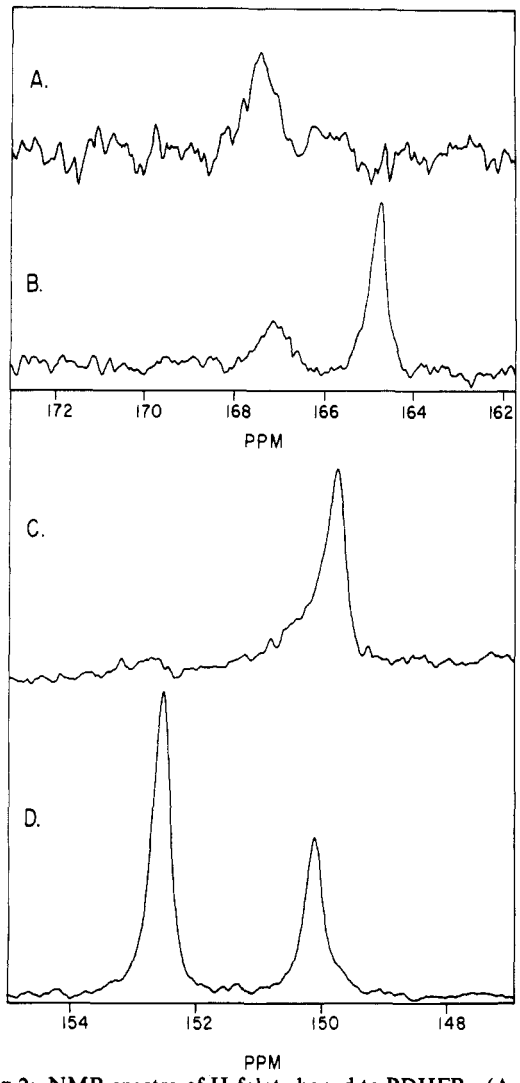

FIGURE 2: NMR spectra of H_2folate bound to BDHFR: (A and B) ^{15}N spectra for [5-^{15}N]H_2folate (131 600 and 89 300 transients, 5-Hz line broadening); (C and D) ^{13}C spectra for [6-^{13}C]H_2folate (99 300 and 70 800 transients, 10-Hz line broadening). Ligand to enzyme ratios were (A) 0.7, (B) 2.1, (C) 0.7, and (D) 2.1. Enzyme concentrations were 0.92 mM (A and C) and 0.80 mM (B and D), and the pH was 7.3. (A and B) 32 K, 540-ms acquisition time, and 0.4-s interpulse delay. (C and D) 32 K, 410-ms acquisition time, and 0.2-s interpulse delay.

(in the presence of enzyme) at the same pH. This is a somewhat smaller displacement of the resonance than in the case of the monoglutamate but in the same direction.

Shift of Bound [6-^{13}C]Folate. Neither the 6-^{13}C nor the 5-^{15}N labeling is ideal for examining whether preprotonation of this substrate occurs during the catalytic cycle, since in the course of enzymic reduction protonation is presumed to occur at N-8. It is also extremely difficult to determine the effect of protonating N-8 on the shift of nuclei at any position in the pteridine ring because N-8 is very weakly basic with a pK_a less than those for N-1 and N-5. The pK_a value of about 2.6 is due to protonation of N-1 rather than N-8. Consequently, the measurements of shift displacement for bound folate and $PteGlu_5$ were undertaken primarily for comparison with the results for the dihydro compounds.

As may be seen from Figure 3 the spectrum for [6-^{13}C]folate and BDHFR in 0.7:1 molar ratio showed only a single peak with a shift of 147.28 ppm. When the ligand to enzyme ratio was increased to 2.1, the spectrum exhibited two peaks with shifts of 147.3 and 150.0 ppm, the former clearly representing bound folate and the latter unbound. Binding to the enzyme

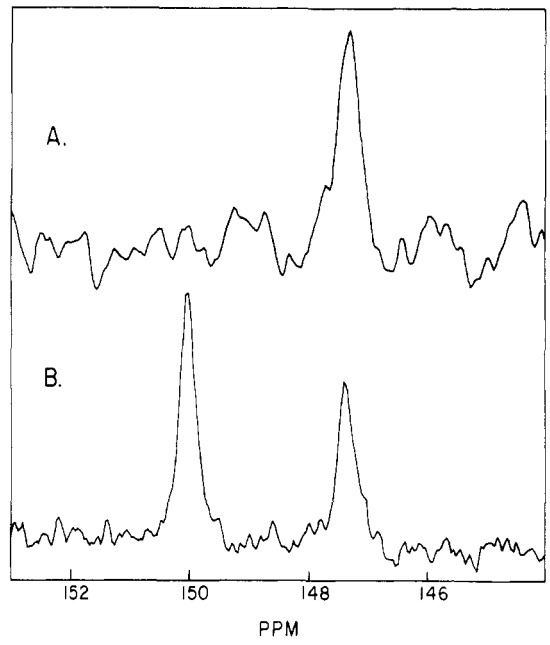

FIGURE 3: ^{13}C NMR spectrum of [6-^{13}C]folate in the presence of BDHFR (0.69 mM) at pH 7.3. The folate to enzyme ratio was 0.7 for (A) and 2.1 for (B) [16 K, 205-ms acquisition time, 44 200 transients, 0.2-s interpulse delay, and 20-Hz line broadening for (A) and 74 000 transients and 10-Hz line broadening for (B)].

produces an upfield displacement of the shift very similar in direction and magnitude to that occurring with [6-^{13}C]H_2-folate.

Effect of the Active Site Carboxyl Group. The significant displacement of the chemical shifts of nuclei in bound H_2biopterin, H_2folate, and folate may be due to local magnetic or electric field effects in the active site arising at least in part from the carboxylate group of Glu^{30}, a conserved residue in the active site of eukaryotic DHFRs and corresponding to a conserved aspartate in bacterial DHFRs. In an attempt to obtain evidence as to the validity of this hypothesis, we examined the effect of lowering the pH to 6.1 on the shift for ^{15}N in bound [5-^{15}N]H_2biopterin. The value obtained, 173.67 ppm, was only slightly less than that obtained at pH 7.3, 174.47 ppm, and still much above the value for the unbound ligand at these pH values (171.18 ppm).

Methotrexate is known to bind with its pteridine ring rotated 180° compared with bound folate, and the two ligands have a completely different interaction with the active site carboxyl. It was therefore of interest to examine how binding to BDHFR affected the ^{13}C shift of [6-^{13}C]aminopterin (10-desmethylmethotrexate) as compared with that of [6-^{13}C]folate. As seen in Table II, binding displaces the chemical shift for C-6 in aminopterin downfield, in the opposite direction to that for this nucleus in folate.

Discussion

Rate of Ligand Exchange between Free and Bound States. In the case of all ligands studied, distinct resonances were observed for uncomplexed and enzyme-complexed molecules, consistent with slow exchange on the NMR time scale. For a nominal chemical shift difference of 1 ppm, this corresponds to dissociation rate constants k_{-1} of $\ll 300$ s^{-1} for the ^{15}N-labeled ligands and $\ll 785$ s^{-1} for the ^{13}C-labeled ligands. These limits are consistent with available dissociation rate constant data for human DHFR, an enzyme with which BDHFR has high homology. Thus, the dissociation rate constants for binary complexes of human DHFR are 27 and 12 s^{-1} for folate and H_2folate, respectively (Appleman et al., 1990). It is noted,

Table II: Summary of Chemical Shifts (ppm) of Ligands Labeled with ^{13}C or ^{15}N

ligand	cation[a]	neutral	anion[b]	$\Delta\delta_{An}$[c]	bound	$\Delta\delta_{DHFR}$[d]
[5-^{15}N]H_2biopterin	111.32	170.66	175.00	+4.34	174.39	+3.21
					173.67[e]	+2.48[e]
[5-^{15}N]H_2folate	<164.42	164.50	170.59	+6.09	167.20	+2.43
[6-^{13}C]H_2folate	>153.30	152.87	152.09	−0.78	150.14	−2.45
[5-^{15}N]H_2PteGlu$_5$	<161.93	165.20	170.64	+5.44	167.20[f]	+1.80
[6-^{13}C]folate	153.4[g]	150.00	147.98	−2.02	147.30	−2.70
[6-^{13}C]aminopterin	151.77	148.41	NA[h]	NA	152.85[i]	+4.27

[a] Ligand protonated at N-5, except in the case of folate where protonation is at N-1. [b] Ligand deprotonated at N-3. [c] $\Delta\delta_{An} = \delta_{anion} - \delta_{neutral}$, i.e., the change in chemical shift due to amide group deprotonation. [d] $\Delta\delta_{DHFR} = \delta_{bound} - \delta_{free}$. The δ_{free} value obtained with enzyme present was used, and this frequently differed slightly from the value determined in absence of enzyme (Figure 1 and column 3) possibly due to greater aggregation of the free ligand at the higher concentration used in the absence of enzyme. Upfield displacements are negative and downfield displacements positive. [e] At pH 6.1. [f] [BDHFR] = 0.5 mM. [g] Cationic shift corresponds to pentaglutamate form; value for folate could not be determined due to limited solubility. [h] NA, not applicable since aminopterin lacks the N-3/C-4–O group. [i] [BDHFR] = 0.6 mM.

however, that additional broadening observed in the ^{15}N NMR studies on the H_2biopterin–DHFR complex (line width ~ 70 Hz) may reflect chemical exchange broadening in this system. A spectral simulation of the data reproduced the observed line widths corresponding to $k_{-1} \approx 100\ s^{-1}$. This higher dissociation rate constant is consistent with the greater dissociation constant of this substrate (Table I).

NMR Spectra of Bound Substrates and Preprotonation. ^{15}N NMR studies of [5-^{15}N]folate complexed with *Lactobacillus casei* DHFR have revealed the existence of two slowly interconverting structures in the binary complex and three in the enzyme–NADP–complex (Birdsall et al., 1987). Subsequent 2D NMR studies have indicated that one of these structures is related to the other two by a 180° rotation of the pteridine ring moiety (Birdsall et al., 1989), so that one bound conformation is presumably similar to that determined crystallographically for the folate–human DHFR binary complex (Oefner et al., 1988) while the others resemble that of the bound inhibitor methotrexate. In contrast with these results and subject to the signal/noise limits of the individual spectra, only single ^{15}N or ^{13}C resonances were observed in any of the binary complexes examined in the present studies. Hence, the bovine enzyme appears to exhibit a greater selectivity for the "folate"-type orientation of the pteridine ring.

As noted in the introduction, one of the principle motivations for the present series of studies was the determination of whether and to what extent N-5 of substrates becomes protonated when they bind to the enzyme. In none of the studies reported here did the NMR spectrum of a bound ligand give any indication that N-5 of the ligand had received a positive charge, or even a small fraction of a positive charge. In particular, the ^{15}N chemical shifts of N-5-labeled substrates are extremely sensitive to N-5 protonation and provide unequivocal evidence against N-5 protonation. As in the case of [5-^{15}N]folate bound to *L. casei* DHFR (Birdsall et al., 1987), the change in chemical shift was downfield on binding whereas protonation caused a large (≈60 ppm) upfield shift for dihydrobiopterin. Similar protonation shifts are expected for N-5 of H_2folate and H_2PteGlu$_5$. The nitrogen resonance of pyridine shifts from 317 to 215 ppm upon protonation, for example (Levy & Lichter, 1979). It may be noted (Table II) that the chemical shifts of N-5 and C-6 which result from complexation with BDHFR are in all cases in the same direction as the shifts which result from deprotonation of the N-3/C-4–O amide group of the pteridine ring. If the active site Glu^{30} side chain is positioned close to N-3 and the C-2 amino group of bound substrates as in the crystal structure of the rHDHFR–folate complex (Oefner et al., 1988) and if the Glu^{30} has a pK of about 6.5 as has previously been proposed for bacterial DHFR (Morrison & Stone, 1988; Fierke et al., 1987), so that it would be primarily in the anionic state at pH 7.2 at which the NMR studies were carried out, then a transfer of a proton from N-3 of the substrate to Glu^{30} might well occur. However, small shifts resulting from enzyme complexation can reflect contributions from nearby resides such as aromatic side chains, as well as changes in ionization state. For the compounds evaluated in Table II, the relatively small magnitude of the shift of N-5 or C-6 resulting either from binding to the enzyme or from amide deprotonation therefore precludes a more definite conclusion about whether the amide group is deprotonated in substrates complexed with BDHFR. It is also uncertain whether the shift change on complexation is consistent with enol formation according to the model of Taira et al. (1987) or with the charge distributions calculated by Uchimari et al. (1989) for this model, but this appears to be possible.

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Exchange and Flip-Flop of Dimyristoylphosphatidylcholine in Liquid-Crystalline, Gel, and Two-Component, Two-Phase Large Unilamellar Vesicles†

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ABSTRACT: The rate and extent of spontaneous exchange of dimyristoylphosphatidylcholine (DMPC) from large unilamellar vesicles (LUV) composed of either DMPC or mixtures of DMPC/distearoylphosphatidylcholine (DSPC) have been examined under equilibrium conditions. The phase state of the vesicles ranged from all-liquid-crystalline through mixed gel/liquid-crystalline to all-gel. The exchange rate of DMPC between liquid-crystalline DMPC LUV, measured between 25 and 55 °C, was found to have an Arrhenius activation energy of 24.9 ± 1.4 kcal/mol. This activation energy and the exchange rates are very similar to those obtained for the exchange of DMPC between DMPC small unilamellar vesicles (SUV). The extent of exchange of DMPC in LUV was found to be approximately 90%. This is in direct contrast to the situation in DMPC SUV where only the lipid in the outer monolayer is available for exchange. Thus, transbilayer movement (flip-flop) is substantially faster in liquid-crystalline DMPC LUV than in SUV. Desorption from gel-phase LUV has a much lower rate than gel-phase SUV with an activation energy of 31.7 ± 3.7 kcal/mol compared to 11.5 ± 2 kcal/mol reported for SUV. A defect-mediated exchange in gel-phase SUV, which is not the major pathway for exchange in LUV, is proposed on the basis of the thermodynamic parameters of the activation process. Surprisingly, the rates of DMPC exchange between DMPC/DSPC two-component LUV, measured over a wide range of compositions and temperatures, were found to exhibit very little dependence on the composition or phase configuration of the vesicles. Evidence is given for defect-mediated exchange from two-component gel phases. The fraction of DMPC exchanging between two-component vesicles indicated fast flip-flop relative to exchange at many, but not all, compositions and temperatures at or above 28 mol % DMPC. Although DMPC exchange between LUV composed of 0.1 mol % DMPC in DSPC has desorption rates equal to those at the other compositions, the flip-flop rates at this composition are at least an order of magnitude smaller.

In biological membranes, the hydrophilic and hydrophobic moieties of the component lipid and protein molecules are strictly segregated. A number of processes occur spontaneously, however, that violate this segregation. These processes include spontaneous interbilayer lipid exchange, transbilayer movement of lipids (flip-flop), insertion into the bilayer of polypeptides, membrane fusion, and bilayer permeation by small polar molecules. Dynamic or static structural defects in the membrane organization have been suggested to be the loci for these processes (Marsh et al., 1976; DeKruijff & Van Zoellen, 1978; Shaw & Thompson, 1982; Wong & Thompson, 1982; Scotto & Zakim, 1988).

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