DIHYDROFOLATE REDUCTASE FROM A METHOTREXATE-RESISTANT *ESCHERICHIA COLI:* PROTON MAGNETIC RESONANCE STUDIES OF COMPLEXES WITH FOLATE AND METHOTREXATE.

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Received September 1,1975

SUMMARY

Proton magnetic resonance studies of 1:1 complexes of *E. coli* dihydrofolate reductase with folate and methotrexate were performed. A resonance at 1850 Hz in 1:1 enzyme-folate was assigned as the C-7 proton of bound folate by comparison with the spectra of enzyme complexed with folate specifically deuterated at C-7. The first order rate constant for folate dissociation was calculated to be less than 110 sec^{-1}. Four of the five histidine residues exhibited the same pK's and chemical shifts in the two complexes with pK values of 8.0, 7.3, 6.5 and ∿5. However, one histidine increased its pK by 0.7 units (6.25→6.95) and its C-2 proton resonance shifted upfield 50 Hz when folate was substituted for methotrexate. Comparison of these results with those of chemical modification and ultraviolet difference spectroscopy experiments suggests that this histidine may be in the folate binding site - possibly near the pteridine portion of that site.

The enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate:TPN^+ oxidoreductase E.C.1.5.1.3) has been identified as the molecular site of action of a number of clinically useful drugs *(1-4)* such as the antineoplastic and immunosuppressive drug methotrexate *(5)*. We have previously reported the large-scale purification of dihydrofolate reductase from a methotrexate-resistant strain of *Escherichia coli B (6,7)*. The relatively low molecular weight, 17,600 daltons, and high solubility in certain ligation states makes it suitable for proton magnetic resonance studies *(8)*. The primary sequence of the protein has been determined *(9)*.

Presented in this article are 220 MHz proton magnetic resonance (pmr) studies of 1:1 complexes of enzyme with folate and methotrexate. These studies identify one histidine residue of five *(9)* which significantly changes its pK between the two complexes. Previous studies of substrate analogs and coenzyme binding to *L. casei* dihydrofolate reductase *(10,11)* have appeared.

MATERIALS AND METHODS

Dihydrofolate reductase was purified from *E. coli* MB 1428 as described previously *(6,7)*. Folic acid dihydrate was purchased from Cyclo, methotrexate from Nutritional Biochemicals, bis-Tris 2,2-bis(hydroxymethyl)-2,2',2''-nitrilo-

triethanol from Aldrich, and sodium-3-trimethylsilyl-(2,2,3,3-^{2}H)propionate from Merck Sharp & Dohme of Canada. All other chemicals were reagent grade.

Folic acid deuterated at C-7 was prepared by dithionite reduction of folate to dihydrofolate in ^2H_2O according to the method of Futterman *(12)* followed by oxidation by iodine. The dihydrofolate, following reduction in ^2H_2O, was suspended in 0.50 M Tris-HCl pH 8.4 and 1 M KI, and standardized spectrophotometrically. Then two molar equivalents of solid iodine were added to the solution, whose pH was monitored continuously to follow the oxidation. After 10 min the reoxidation was complete and DEAE-Sephadex A-25 (Pharmacia), previously equilibrated with 0.55 M NaCl 0.05 M Tris-HCl pH 7.2 was added to purify the folate. The filtrate was adjusted to pH 2.8, cooled to 0°, and centrifuged. The yellow precipitate was washed four times with 5 mM HCl at 0°. This procedure gave folate in 34% yield which had an ultraviolet absorption spectrum and pmr spectrum essentially identical to that of free folate with the exception that the C-7-H resonance in the pmr spectrum was only 35 ± 5% of its intensity in fully protonated folate. The theoretical intensity would be 50%, thus the iodine reoxidation of dihydrofolate must preferentially remove the proton at C-7. The partially deuterated folate was again reduced and oxidized by the above protocol. The overall recovery of folate was 10%. This material was 18 ± 5% protonated at C-7; a portion of it was used to prepare a complex of enzyme with C-7-^{2}H folate as described below.

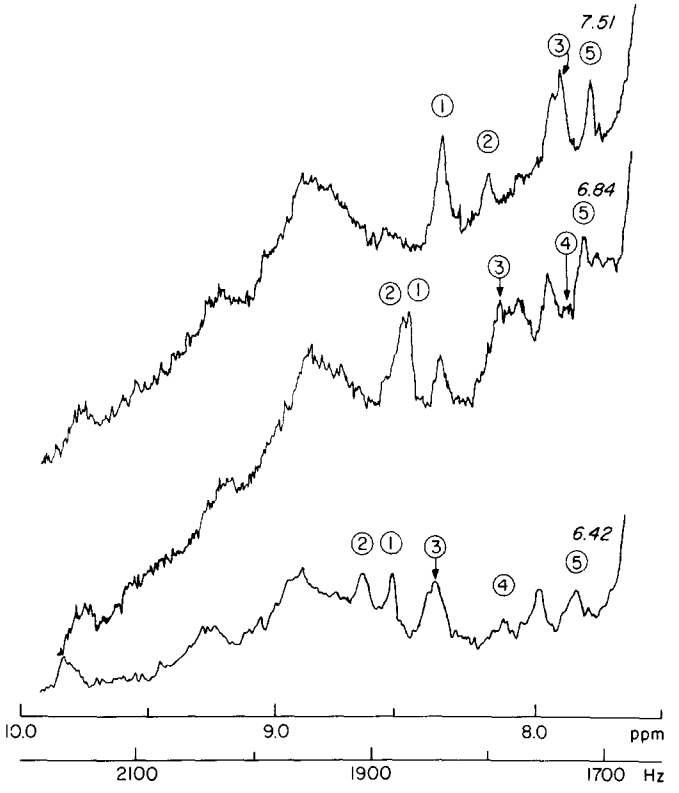

FIGURE 1: Lowest field portion of pmr spectra of 1:1 folate:dihydrofolate reductase at pH 7.51 (top), 6.84 (middle), and 6.42 (bottom). Enzyme, 4 mM; T = 25°; buffer, 0.10 M NaCl, 5 mM bis-Tris HCl. The five histidine C-2-H protons are labelled ① to ⑤.

Solutions for pmr spectra were prepared by addition of 1.2 - 1.5 equivalents of ligand to 50-100 ml of a roughly 0.5 mg/ml enzyme solution, followed by ultrafiltration to about 10 mg/ml and then lyophilization. The resulting powder was dissolved in 0.45 - 0.50 ml ^2H_2O and dialyzed twice versus 50 ml 0.10 M NaCl 0.005 M bis-Tris ^2HCl in ^2H_2O at the desired pH. Since folate has been shown *(13)* to be slowly degraded at 25° by *E. coli* MB 1428 dihydrofolate reductase, the enzyme-folate solutions were stored at -20°.

All pmr spectra were obtained on a Varian 220 MHz pmr spectrometer; the signal-to-noise characteristics of enzyme spectra were improved with a computer of average transients. All spectra were run at 25° and internally referenced to the methyl resonance of sodium 3-trimethyl-(2,2,3,3-^2H)propionate in units of Hz or parts-per-million (ppm) with downfield shifts assigned positive values. All pH measurements reported are direct electrode readings. pH measurements on pmr solutions were made before and after spectral accumulation and the spectra were disregarded unless the two measurements agreed within ±0.04.

RESULTS

The IUPAC-IUB *(14)* numbering system for folates was used. The dissociation constants for binding of methotrexate and folate to dihydrofolate reductase at pH 7.2, 0.10 M ionic strength, are 0.004 μM *(7)* and 3 μM *(15)* respectively. Hence, there is no appreciable unliganded enzyme in pmr solutions of 2-5 mM enzyme and equimolar ligand.

Typical spectra of the aromatic region of 1:1 enzyme-folate are shown in Figure 1. The pH of the solution is indicated above each system. Below pH 6, the enzyme-folate and methotrexate complexes are unstable and slowly precipitate; hence, lower pH data could not be obtained. The large number of normally exchangeable protons in the enzyme-folate complex indicates the presence of amide protons inaccessible to solvent.

The titration curves for the resonances of 1:1 enzyme-folate between 1620 and 1930 Hz are presented in the left side of Figure 2. The resonances indicated with dotted lines correspond to exchangeable protons. The dependence of the resonances labelled 1 through 5 in Figure 2 upon pH was typical of histidine protons, and their chemical shift and chemical shift change upon protonation indicated that these were C-2 histidine protons *(16)*. The curves shown for histidines 1, 2 and 4 in Figure 2 were visual fits to a standard histidine C-2 proton titration curve, and gave the pK values tabulated in Table I. Histidine 5 was just beginning to titrate at pH 6 so that pK could only be estimated to be very low, $\sim$5, if a typical titration curve was assumed. Curve 3 may have exhibited a somewhat larger than normal chemical shift change

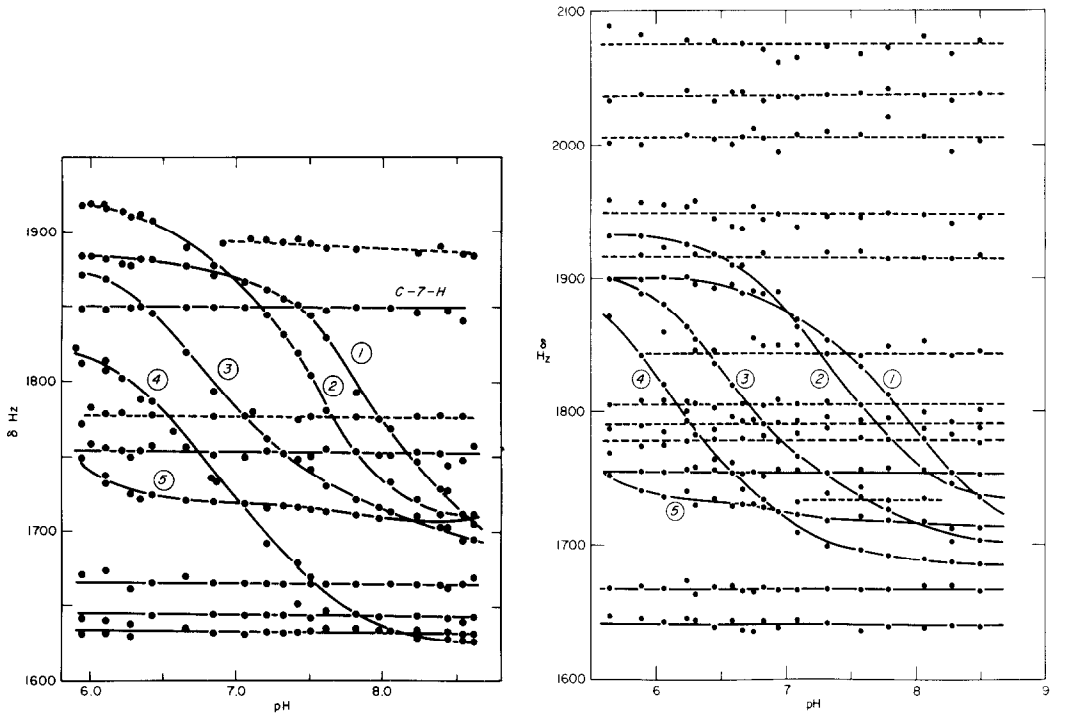

FIGURE 2: Dependence of chemical shift upon pH for the lowest-field resonances of 1:1 enzyme-folate (left side) and 1:1 enzyme-methotrexate. Histidine C-2-H resonances labelled ① to ⑤ . Resonance at 1850 Hz on left is the folate C-7-H. Dotted lines represent slowly exchangeable resonances.

TABLE I. *Histidine pK values. All at 25° and 0.1 ionic strength.*

Resonance	Enzyme Folate	Enzyme Methotrexate
1	8.0 ± 0.1	8.0
2	7.3	7.35
3	6.4	6.55
4	6.95	6.25
5	∿5	∿5

upon protonation, but overlap with non-titrating resonances at acidic and basic pH made the exact endpoints difficult to determine; it was fitted using experimentally derived titration endpoints. The resonance of the C-2 proton

of histidine 4 often overlapped more intense resonances and appeared to be broader than the other four histidine C-2 resonances. When this histidine was unprotonated, its C-2-H exhibited an abnormal chemical shift, being 0.4 ppm to high field of the position for such a resonance in an extended random-coil polypeptide *(17)*.

The preparation of folic acid specifically deuterated at C-7 was used to identify the C-7 proton in the enzyme-folate complex. The upper spectrum in Figure 3 was traced from a spectrum for a complex of reductase with folate, 82% deuterated at C-7, at pH 7.01. The lower spectrum was replotted point-by-point to give comparable scale widths, and corresponds to a spectrum at pH 7.05 of a solution containing enzyme plus fully-protonated folate. This demonstrated that the 1850 Hz resonance (see arrow) corresponds to the C-7 proton of bound folate. The identity of the non-exchangeable proton resonance at 1754 Hz is unknown.

The titration curves for the resonances of 1:1 enzyme-methotrexate between 1600 and 2100 Hz are shown in the right side of Figure 2. Exchangeable protons are indicated by dotted lines. The resonance at 1850 Hz was not observed below about pH 6.7, possibly due to resonance breadth, but not due to exchange for deuterium, since this resonance reappeared when the pH of the solutions was raised to basic pH. As with enzyme-folate, there were five histidine C-2 resonances. The solid lines connecting the resonances of histidine 1 and 2 were visual fits of the data points to a standard histidine C-2-H titration curve and gave the pK values summarized in Table I. Once again, histidine 5 had an abnormally low pK which could only be estimated. Histidine 3 and 4 exhibited somewhat larger chemical shift changes upon protonation, and were fitted using experimentally derived titration endpoints.

In both enzyme-methotrexate and enzyme-folate all five of the histidine residues are in nonequivalent environments. Four of the five histidine C-2 resonances exhibit a high degree of homology for the two complexes in chemical shift and pK; however, the C-2-H resonance of histidine 4 shifts about 50 Hz

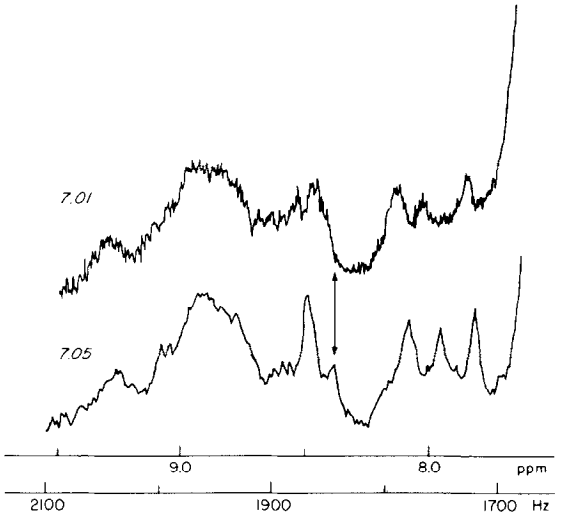

FIGURE 3: Comparison of lowest-field portion of aromatic region of pmr spectra of enzyme:folate and enzyme:specifically deuterated folate. Upper trace: 1:1 enzyme:folate 82% deuterated at C-7; pH 7.01; 2.7 mM. Lower trace: 1:1 enzyme:folate; pH 7.05; 4.4 mM. Both spectra at 25° in 0.10 M NaCl 0.005 M bis-Tris-^{2}HCl. The arrow indicates the resonance position for C-7-H of bound folate.

upfield and its pK increases by 0.7 units when folate is substituted for methotrexate. The enzyme-methotrexate complex exhibits a larger number of slowly-exchangeable protons than does enzyme-folate; this could reflect the tighter binding of methotrexate compared to folate.

DISCUSSION

The C-2-H histidine resonances provide very sensitive probes of enzymes since their chemical shifts and pK's reflect their magnetic and chemical environments. The homology of four of the five histidines in the enzyme-folate and methotrexate complexes suggests the gross conformation of the enzyme is similar for the complexes. The change in pK and chemical shift of histidine 4 might implicate this histidine in the folate site, however, such alterations might also be caused by a localized conformational change. Previous chemical modification experiments *(18,19)* have demonstrated the importance of histidine in maintaining the active configuration of the enzyme, and it was found that one histidine is protected from reaction with ethoxyformic anhydride by either

folate or methotrexate *(18)*. If this histidine is actually in the folate site, one might speculate further on its location. Ultraviolet difference experiments indicated that bound methotrexate bears an extra positive charge on the pteridine ring while folate does not *(20)*. The magnitude and sign of the difference in pK of histidine 4 in enzyme-methotrexate and enzyme-folate are consistent with that histidine being proximal to a positive change. The extra positive charge on methotrexate could also be responsible for the downfield chemical shift of histidine 4 in enzyme-methotrexate compared to enzyme-folate *(21,22)*.

When excess folate is added to the enzyme, resonances corresponding to free and bound ligand are observed, 1900 Hz *(23)* and 1850 Hz, respectively, indicating that folate is in slow exchange. The rate constant for dissociation of ligand to the free form is therefore less than about $2\pi \Delta\delta/2\sqrt{2}$, where $\Delta\delta$ is the change in chemical shift between free and bound ligand *(21)*. Since $\Delta\delta$ is 50 sec^{-1} here, the rate constant must be less than 110 sec^{-1}. This rate is somewhat slower than the maximal turnover rate of 675 ± 100 sec^{-1} for the reduction of dihydrofolate to tetrahydrofolate by the enzyme under analogous conditions *(7)*, but is considerably above the rate of reduction of folate which is roughly 1000-times slower than dihydrofolate reduction (unpublished results). Using the dissociation constant for the enzyme-folate complex of 3 μM at 25° *(15)* and the above upper limit for the dissociation rate constant, the second order rate for folate binding must be less than 110 $sec^{-1}/3 \pm 1$ μM, or $3.7 \pm 1.3 \times 10^7$ M^{-1} sec^{-1}.

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