INTERACTION OF ^{13}C-ENRICHED FOLATE WITH DIHYDROFOLATE REDUCTASE STUDIED BY CARBON MAGNETIC RESONANCE SPECTROSCOPY

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Summary: In the presence of dihydrofolate reductase the carbon magnetic resonance spectrum of folate labeled at the benzoylcarbonyl carbon with ^{13}C contains two broadened peaks arising from free and enzyme-bound folate, the latter appearing over 2 ppm upfield from free folate. Addition of TPN^+ causes sharpening of both peaks indicating formation of a single folate-TPN^+-enzyme ternary complex. Methotrexate specifically displaces folate from the ternary complex regenerating a single sharp resonance at 170.4 ppm characteristic of free folate. Line width changes show that folate is bound more tightly in the ternary than in the binary complex. Increased shielding of this carbonyl upon binding is inconsistent with its participation in a H-bond.

INTRODUCTION

This laboratory has described previously in detail the large changes induced in the proton magnetic resonance (PMR) spectrum of dihydrofolate reductase apoenzyme by substrates (folate, dihydrofolate), cofactors (TPN^+, TPNH) and the potent inhibitor, methotrexate, and established the efficacy of this probe in studying the conformational responses of this protein to various ligands (1, 2). More recently we have extended these studies to a large number of diverse structures including substrates as well as inhibitors which are believed to bind to the dihydrofolate site (3). It proved possible to readily differentiate the enzyme-folate binary complex from complexes formed with virtually all other ligands including such close structural analogs as 10-methyl- and 4-amino-4-deoxy folate (aminopterin). Such data demonstrate the sensitivity of the method and provide the basis for determining the molecular basis of the enzyme conformational responses to the various ligands including important chemotherapeutic agents, e.g., methotrexate and trimethoprim. Relatively less information can be obtained about the bound ligands themselves from PMR spectra due to interference by the proton resonance signals arising from the protein and their own broadened signals in the bound state.

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This problem may be overcome by using ligands specifically enriched with ^{13}C in conjunction with Fourier transform carbon magnetic resonance (CMR) spectroscopy. The chemical shift spread of ^{13}C is 20 fold greater than that for protons, and good resolution may be obtained from proton decoupled spectra for even highly immobilized carbon nuclei (4). This report describes the first use of folate specifically enriched with ^{13}C to study the mechanism of its binding to dihydrofolate reductase alone and in the presence of TPN^+ and methotrexate.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: TPN^+, Calbiochem, La Jolla, Ca.; folate, Sigma Chemical Co., St. Louis, Mo.; deuterium oxide (99.8 per cent), Stohler Isotope Chemicals, Rutherford, N.J. and methotrexate was a gift from Lederle, Pearl River, N.Y. Folate labeled at the benzoylcarbonyl carbon with ^{13}C (90 atoms per cent excess) was prepared and characterized by the procedure of Plante et al. (5). The location of this label is shown in Figure 1. Dihydrofolate was prepared by the method of Futterman (6) as modified by Blakley (7). Protein was determined by the microbiuret method of Goa (8). Dihydrofolate reductase activity was determined spectrophotometrically by the method of Osborn and Huennekens (9) as modified by Pastore and Friedkin (10).

Enzyme purification. Dihydrofolate reductase was obtained from a methotrexate resistant strain of Lactobacillus casei developed by Crusberg et al. (11) and purified to homogeneity by affinity chromatography using pteroyllysine-agarose as described previously (12). The apoenzyme used in these studies catalysed the reduction of 15 μmoles of 7,8-dihydrofolate per minute at ambient temperature. Ultraviolet and PMR spectroscopy showed that the enzyme was free of bound folate and pyridine nucleotide coenzymes.

Carbon magnetic resonance spectroscopy. Enzyme used for CMR spectroscopy was adjusted to a concentration of 1.3 mM in 0.05 M potassium phosphate-0.5 M KCl, pH 6.8, containing 5 per cent D_2O for field frequency lock and maintained at 12°. Fourier transform CMR spectra were obtained with a JEOL PS-100 spectrometer operating at 25.03 MHz. Data acquisition and processing were done by a Nicolet 1085 computer. The acquisition time was 0.8192 sec (8,192 data points), and the 7 μsec pulses were repeated every 0.92 sec. Chemical shifts were measured relative to 10 mM internal $^{13}CH_3OH$ and referred to tetramethylsilane (TMS) by adding 49.8 ppm.

RESULTS

The spectra in Figure 2 were obtained from an enzyme sample containing a fixed amount of $^{13}CH_3OH$; therefore, neglecting possible changes in T_1's and nuclear Overhauser effects, comparison of resonance amplitude ratios of the

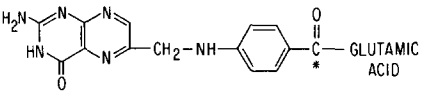

Figure 1. Structure of folate; an asterisk (*) indicates the benzoylcarbonyl carbon.

two ^{13}C-enriched compounds provides an approximate measure of line broadening experienced by folate in its various complexes. Note that spectra (a) and (b) contain 1.3 mM ^{13}C-folate while (c), (d) and (e) contain 2.6 mM ^{13}C-folate. The CMR spectrum of 1.3 mM ^{13}C(benzoyl carbonyl)-folate is shown in Figure 2(a). The chemical shift, 170.4 ppm relative to TMS, compares with a value of 169.6 ppm obtained for this position from the natural abundance spectrum of 0.2 M folate by Lyon et al. (13), and their more shielded value might be accounted for in part by the strong intermolecular ring stacking interaction of folate which would occur at this high concentration and pH (14).

In the presence of an equivalent amount of dihydrofolate reductase apoenzyme the folate benzoyl carbonyl resonance signal is broadened and shielded, appearing at 168.0 ppm in Figure 2(b). The low rise in the base line just to the left of this peak is due to the natural abundance ^{13}C resonances of the protein carbonyl carbons. Addition of a second equivalent of ^{13}C-folate gives a spectrum having two broadened folate peaks appearing at 167.6 and 170.1 ppm in Figure 2(c) which are assigned to bound and free folate, respectively. The chemical shift difference between the bound forms in Figure 2(b) and (c) is not judged to be significant because of the relatively low

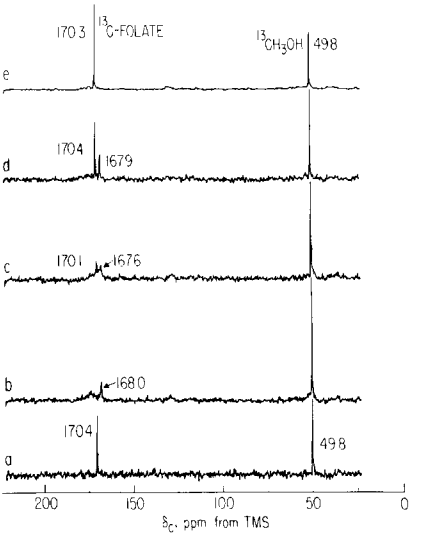

Figure 2. CMR spectra at 25.03 MHz of ^{13}C-enriched folate: (a) 1.3 mM, 4000 transients; (b) 1.3 mM plus 1.3 mM dihydrofolate reductase, 36,000 transients; (c) 2.6 mM plus 1.3 mM enzyme, 16,000 transients; (d) 2.6 mM plus 1.3 mM enzyme and 2.6 TPN^+, 16,000 transients; (e) 2.6 mM plus 1.3 mM enzyme, 2.6 mM TPN^+ and 1.5 mM methotrexate, 40,000 transients. Internal standard is $^{13}CH_3OH$. All spectra run at 12°.

signal-to-noise ratio and the line width of the latter peak.

The line widths of both broad peaks arising from the folate-enzyme binary complex are greatly reduced when 2 equivalents of TPN^+ are added to form the folate-TPN^+-enzyme ternary complex, shown in Figure 2(d), while their chemical shifts remain essentially the same. Addition of methotrexate to the folate-TPN^+-enzyme ternary complex causes the two peaks to coalesce into a single very sharp peak at 170.3 ppm, Figure 2(e), which is the chemical shift of folate in the absence of binding interactions. This result is consistent with the fact that methotrexate binds to the enzyme more tightly than folate (15) and thus would be expected to replace folate in the ternary complex (2).

DISCUSSION

The appearance of two broadened resonance peaks arising from ^{13}C (benzoyl carbonyl)-folate in the presence of L. casei dihydrofolate reductase provides unique information about the molecular basis and dynamic characteristics of the binding process. With respect to the binding mechanism the 2.4 ppm upfield chemical shift change experienced by the benzoylcarbonyl carbon in the bound form is inconsistent with H-bond formation at this position since such interaction would cause a change opposite to the one observed (17); however, the change would be in accord with disruption of existing H-bonding between the benzoyl carbonyl oxygen and the aqueous solvent upon transfer of the ligand to a hydrophobic environment on the enzyme. The increased shielding of the carbonyl could also be accounted for by a stacking type interaction between the benzene ring of folate and an aromatic amino acid of the protein. Independent evidence obtained by PMR spectroscopy has shown that folate does interact strongly in this manner associating both with itself (14) and even more strongly with tryptophan (18). The upfield chemical shift changes caused by ring stacking interactions can be larger for ^{13}C than for protons (19) and could account for the shift changes of approximately 2 ppm observed here. It should be noted that other possible interaction mechanisms could explain the observed upfield shift, e.g., electrostatic interaction of the ligand with charged groups on the enzyme or distortion of the amide bond upon binding.

Regarding binding dynamics the observation of two well resolved peaks corresponding to free and bound folate indicates that exchange between the two forms (folate + enzyme $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ folate-enzyme) is slow on the NMR time scale; from the observed peak separation of 60 Hz the lifetime of the folate-enzyme binary complex must be $>>5 \times 10^{-3}$ sec. The sharpening of both free and bound peaks on going to the folate-TPN^+-enzyme ternary complex shows that

the ternary complex is much longer lived than the binary complex. Assuming no exchange contribution to the ternary complex line width, the difference between the ternary and binary complex line widths ($\Delta\nu$ = 6 ± 2 Hz) gives the binary complex dissociation rate constant k_{-1} = 19 ± 6 sec^{-1}. The decreased exchange rate of folate in the ternary complex is consistent with independent observations which show that dihydrofolate site-specific analogs are bound more tightly to the enzyme in the presence of cofactors (20).

It is of interest to compare these results with those of Way et al.(21) who studied binding of ^{13}C(nicotinamide carboxamido)-TPN^+ to L. casei dihydrofolate reductase. They observed two resonances shifted well upfield from free TPN^+ for the folate-TPN^+-enzyme ternary complex and they interpreted their results as evidence for two different forms of the ternary complex. Our results provide evidence for a single form of this ternary complex. Further studies using ^{13}C labelling at other positions in folate as well as TPN^+ may resolve this apparent conflict.

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