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^{15}N NMR studies of the conformation of *E. coli* dihydrofolate reductase in complex with folate or methotrexate

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We have employed ^{15}N NMR to characterize the conformations of *Escherichia coli* dihydrofolate reductase (ECDHFR) in complex with [5-^{15}N]folate or [5-^{15}N]methotrexate (MTX). Two ^{15}N resonances were observed for DHFR/MTX binary complex. The relative population of these two conformations is pH dependent. Addition of $NADP^+$ or NADPH results in the disappearance of the low field resonance. In contrast, only one conformation was observed for both the DHFR/folate and DHFR/folate/$NADP^+$ complexes. However, the ^{15}N chemical shift of [5-^{15}N]folate in the binary DHFR/folate complex is 7.28 ppm upfield from that of the ternary complex, suggesting the possible loss of a hydrogen bonding to N5 of folate in the ternary complex.

Dihydrofolate reductase; Methotrexate; Folate; NMR; $NADP^+$

1. INTRODUCTION

Dihydrofolate reductase (DHFR) (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Further derivatization of tetrahydrofolate provides a number of coenzymes involved in one-carbon transfer reactions including those important for the biosynthesis of purines and thymidylate [1]. Furthermore, DHFR is an enzyme of considerable pharmacological interest. It is the target of several antifolate drugs such as the antitumor agent MTX and the antibacterial drug trimethoprim (TMP). The inhibitory pattern of these antifolates varies considerably from species to species. Thus, considerable efforts have been directed toward determining the structural basis of this species variations.

The structures of ECDHFR and its complexes with folates and MTX have been the subjects of intensive studies by several physical methods, including kinetics [2,3], X-ray crystallography [4–7], and NMR [8,9]. The apoenzyme and its binary complex with MTX were shown to exist in 2 conformations [2,4,7–9] whilst the binary complex of ECDHFR/folate and the ternary complex of ECDHFR/MTX/NADPH appeared to exist in a single conformation [7,9]. X-ray crystallography and NMR studies further demonstrated that in the DHFR complex, the conformation of MTX has the pteridine ring roated by ca. 180° relative to that of folate [5–7]. However, the structural heterogeneity appears to be species specific. Selinsky et al. found that folate binds to bovine DHFR in a single conformation [10]. In contrast, folate appears to exist in 2 conformations in the binary complex with *Lactobacillus casei* DHFR (LCDHFR) and in 3 conformations in the ternary complex with LCDHFR and $NADP^+$ [11]. In this communication we report the results of ^{15}N NMR experiments aimed at characterizing the conformations of ECDHFR in binary and ternary complexes with folate, MTX, and $NADP^+$.

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2. MATERIALS AND METHODS

ECDHFR was isolated from a DHFR over-producing *E. coli* strain carrying multiple copies of the pUC8 plasmid. Enzyme was isolated and assayed as described previously [12]. Enzyme purity was found to be better than 95% as checked with the denaturating SDS-PAGE method. Protein concentration was determined by the method of Bradford using Pierce protein assay reagent (Pierce Chemical Co.). [5-^{15}N]folate was prepared from 2,4,6-triaminopyrimidine by nitrosation using $Na^{15}NO_2$ (Isotech) and reduction of the nitroso compound with Raney-Ni/H_2 to yield [5-^{15}N]2,4,6-tetraaminopyrimidine. The reaction product mixture was reacted with 1,3-dihydroxy-acetone according to the method of Konrad and Pfleiderer [13] to yield [5-^{15}N]-2,4-diamino-6-(hydroxymethyl)pteridine. The (hydroxymethyl)pteridine was converted to [5-^{15}N]-2,4-diamino-6-(bromomethyl)pteridine hydrobromide by the method of Piper and Montgomery [14] except that dry CH_2Cl_2 was used without the formation of hydrobromide intermediate. The product of this reaction was allowed to react with N-(4-aminobenzoyl)-L-glutamic acid in dimethyl acetamide to form [5-^{15}N]aminopterin according to the method of Piper and Montgomery [14]. [5-^{15}N]folate was obtained according to Selinsky et al. [10]. [5-^{15}N]MTX was prepared by reacting aminopterin with 38% HCHO and NaBHCN at pH 6.5 and room temperature. The purified final product showed a single UV-absorption spot and same R_f as the

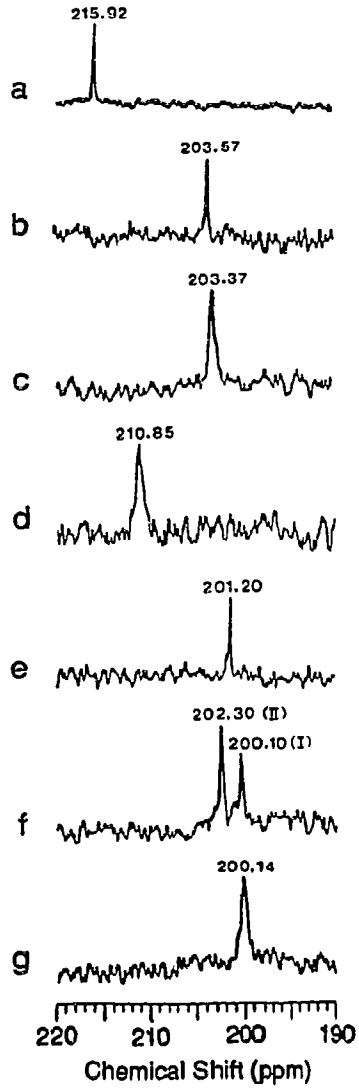

Fig. 1. ^{15}N NMR spectra of (a) [5-^{15}N]folate in DMSO; (b) [5-^{15}N]folate in buffer; (c) [5-^{15}N]folate/DHFR; (d) [5-^{15}N]folate/DHFR/NADP$^+$; (e) [5-^{15}N]MTX; (f) [5-^{15}N]MTX/DHFR; and (g) [5-^{15}N]MTX/DHFR/NADP$^+$ in 50 mM potassium phosphate buffer (pH=6.8), 100 mM Kcl, 2 mM EDTA in D_2O. Spectra were obtained at room temperature. Chemical shifts are given on the figure and are referenced to external natural abundant ^{15}N nitrogen in formamide.

authentic standard on a cellulose TLC plate in 50 mM potassium phosphate buffer, pH 7.4.

^{15}N NMR spectra were obtained with a 10 mm NMR tube containing 2.0 ml of 1.5 mM enzyme in 50 mM potassium phosphate, 100 mM KCl, 2 mM EDTA buffer in D_2O. Folate and MTX were added to 0.9 molar equivalent while NADP$^+$ and NADPH were added at 1.0 molar equivalent to form the ternary complexes. PHs of the samples were adjusted using 1.0 M DCl of NaOD and were directly read from an Orion model 811 pH meter equipped with an Ingold 6030-2 electrode. All NMR spectra were obtained with a Varian XL-400 spectrometer operating at 40.547 MHz for ^{15}N. Experimental conditions were: single pulse (45° flipping angle) experiment with WALTZ-16 decoupling; spectral width=11 kHz at 16K data point resolution; pulse interval=1.22 s. Each spectrum is the result of signal averaging of ≈ 120 000 accumulations. Unlabelled formamide in a coaxial tube was used as external standard.

3. RESULTS AND DISCUSSION

Fig. 1 shows a set of ^{15}N NMR spectra of [5-^{15}N]folate and [5-^{15}N]MTX in various protein complexes at pH=6.8. The following prominent features can be discerned from these spectra. (i) A single resonance is observed for DHFR/folate (Fig. 1c) or DHFR/folate/NADP$^+$ (Fig. 1d) complexes, suggesting the presence of a single conformation for these complexes. (ii) For MTX/DHFR binary complex (Fig. 1f) the presence of two resoances at 200. 1 ppm (conformation I) and 202.3 ppm (conformation II) were observed, suggesting that the binary complex of DHFR/MTX exists in 2 conformations. From the linewidth of the ^{15}N resonance we estimate that the rate of interconversion between these 2 conformations must be slower than 30 s^{-1}. The relative population of these 2 conformers at pH=6.8 is P_I:P_{II} = 0.37:0.63. (iii) Addition of NADP$^+$ to the binary complex of DHFR/[5-^{15}N]MTX resulted in the disappearance of the down-field resonance. Thus, the ternary complex of DHFR/MTX/NADP$^+$ prefers to exist in conformation I. (iv) The ^{15}N resonance of [5-^{15}N]folate in aqueous solution (Fig. 1b) is 12.35 ppm up-field of that observed in DMSO (Fig. 1a). This is to be expected since in aqueous environment N5 of folate is likely to form hydrogen bonding with water molecules which would cause the resonance to shift upfield. (v) The ^{15}N chemical shifts of [5-^{15}N]folate and [5-^{15}N]MTX in most protein complexes are near that observed for the free ligands in aqueous solution. This observation suggests that the environments of N5 of folate in the binary complex and of MTX in the binary and ternary complexes are very similar to that found in the aqueous solution. Thus, it is likely that N5 of folate and MTX in these complexes form hydrogen bonds with solvent molecules in the binding pocket. In contrast, the ^{15}N chemical shift of [5-^{15}N]folate/DHFR/NADP$^+$ ternary complex is shifted down-field by 7.28 ppm from that observed for the free [5-^{15}N]folate in aqueous solution. Such a shift is too large to be attributed to ring current shift effect alone due to the presence of NADP$^+$, but is too small to be due to direct protonation of N5 which is estimated to cause an upfield shift of >60 ppm ([10] and Huang et al., unpublished result). Alternatively, the large down-field shift may be caused by the change in hydrogen-bonding network among folate, the surrounding solvent molecules, and the protein moieties. X-ray crystal structure of the ternary complex of DHFR/folate/NADP$^+$ revealed the presence of an elaborated hydrogen-bonding network involving Asp^{27}, two fixed water molecules, Thr^{113} and Trp^{22} [6,7]. However, no fixed water molecule was found to bind directly to N5. Thus, a proton relay scheme involving this hydrogen bonding network and keto-enol tautomerization of the N3–C4 bond was proposed to explain enzyme catalyzed protonation of N5. The pK of this tautomerization was shown to be 8.56 [10]. N3–C4 tautomeriza-

tion causes a change in [5-^{15}N]folate chemical shift of ≈ 3 ppm [10] which is small compared to the 7.28 ppm shift observed due to NADP$^+$ binding. Thus, even though we cannot rule out this possibility, the formation of a hydrogen bonding network involving N3–C4 tautomerization may not be sufficient to induce a chemical shift change of over 7 ppm. A most probable explanation of this large chemical shift is the change in direct hydrogen bonding of solvent molecule to N5. The solvent molecule in this hydrogen bonding may be too mobile to be observed by X-ray crystallographic technique. Since the ^{15}N chemical shift of [5-^{15}N]folate/NADP$^+$/DHFR is still 5.07 ppm upfield of that observed in DMSO the hydrogen bonding in the ternary complex may not be totally lost. Alternatively, the presence of a positive charge in NADP$^+$ may cause substantial chemical shift in the nitrogen resonance. More work is needed to determine the origin of this shift. (vi) The linewidth of the ^{15}N resonances differ substantially among the 4 protein complexes, 30 Hz for [5-^{15}N]folate/NADP$^+$/DHFR (Fig. 1d) and 15 Hz for the binary complex of MTX with DHFR (Fig. 1f). At present we do not know the source of this variation. Change in temperature appeared to have no effect on the linewidth of the spectrum of the folate tenary complex (Fig. 1d). There is also an indication of the presence of a small population of additional components in the binary complex of MTX with DHFR (Fig. 1f), however the poor quality of the spectrum precludes us from making a definite assignment.

To further characterize the conformational equilibrium of MTX/DHFR we have obtained ^{15}N NMR spectra of this complex over a wide range of pH values. Fig. 2 showed a set of ^{15}N NMR spectra of the binary complex, [5-^{15}N]MTX/DHFR, obtained at various pHs. The chemical shifts of the 2 resonances at 200.2 ppm (conformation I) and 202.2 (conformation II) are relatively insensitive to pH variation from pH=6.0 to 8.8. However, the relative intensity of the 2 resonances varies from P_I:P = 1:1 at pH=6.0 to 1:3 at pH=8.8. Thus, high pH favors conformation II. The group(s) that is responsible for the conformational transition has not been identified. However, the titration pattern does not follow a simple Henderson–Hasselbalch behavior, implying that the conformational transition may not be controlled by a single titratable group.

Our results are in good agreement with the NMR results reported by Falzone et al. who observed the presence of a single conformation for folate/DHFR [9] and MTX/DHFR/NADPH [8] complexes and 2 conformations for the apoenzyme and the binary complex of DHFR with MTX [8]. In *Lactobacillus casei* the presence of 2 conformations (I and II) was observed for the binary complex of LCDHFR/[5-^{15}N]folate and 3 conformations (I, IIa and IIb) for the ternary complex of LCDHFR/[5-^{15}N]folate/NADP$^+$ [11,15]. The chemical shift difference between conformation I and II (IIa and IIb) is also ≈7 ppm with conformation I existing in high field. This difference is also attributed to the difference in hydrogen bonding to N5 which is present in conformation I but absent in conformation II. Furthermore, the pteridine ring of folate exists in 2 conformations, the productive and nonproductive conformations, which are related by a 180° rotation about an axis approximately along C2–NH_2 bond [11]. In the *E. coli* system, the folate ring was found to bind in the productive conformation [4,9] while in the binary complex of ECDHFR/MTX the pteridine ring of MTX is found to be oriented in the nonproductive conformation in both conformations [8]. The ring orientation in ECDHFR/folate/NADP$^+$ and ECDHFR/MTX/NADP$^+$ are still unknown. Selinsky et al. observed a single conformation for folate in complex with the bovine enzyme [10]. Thus, in spite of structural homology, the structure details can be substantially different in different species.

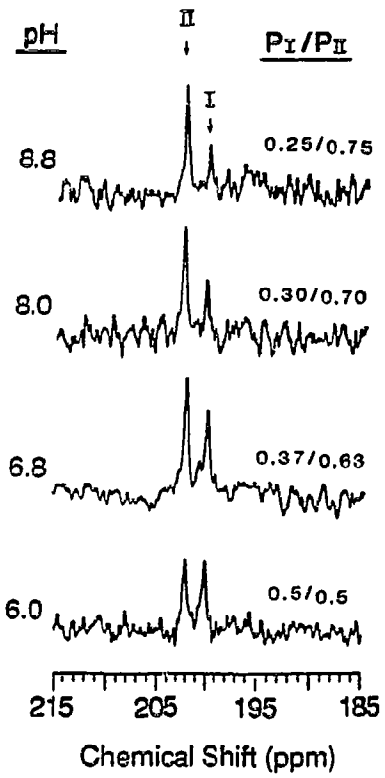

Fig. 2. ^{15}N spectra of [5-^{15}N]MTX/DHFR complex at various pHs as indicated. The relative population of the two conformations, P_I:P_{II} are also given.

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