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The conformations of trimethoprim/*E. coli* dihydrofolate reductase complexes

A ^{15}N and ^{31}P NMR study

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We have employed ^{15}N and ^{31}P NMR techniques to characterize the conformations of trimethoprim (TMP)/*E. coli* dihydrofolate reductase (DHFR) complexes in the presence and absence of NADPH and $NADP^+$. A single conformation was observed for TMP/DHFR, $NADP^+$/DHFR, NADPH/DHFR, and TMP/NADPH/DHFR complexes. In the ternary complex of TMP/$NADP^+$/DHFR both the ^{15}N and ^{31}P spectra revealed the presence of two conformations. However, the conformations of TMP and $NADP^+$ in the ternary complex may not be correlated, resulting in the possible existence of four conformations for the protein ternary complex.

Dihydrofolate reductase; *E. coli*; Trimethoprim; Conformation; NMR, ^{15}N, ^{31}P, $NADP^+$

1. INTRODUCTION

DHFR (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3.) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) [1]. The latter participates in the synthesis of thymidine and some amino acids. Depletion of the H_4F pool results in the cessation of cell growth and can lead to the eventual cell death. Besides its biochemical importance, DHFR is also an important therapeutic target enzyme for a group of antifolate drugs, notably trimethoprim (TMP), methotrexate, and pyrimethamine, for the treatment of cancers, malaria, bacterial infections and other diseases.

High-resolution X-ray crystal structures of binary TMP/DHFR and ternary TMP/DHFR/$NADP^+$(H) complexes from several bacterial and mammalian species have been determined [2,3]. Results of NMR studies have shown that TMP is protonated at N-1 when bound to DHFR from *Lactobacillus casei* [4], bovine liver [5], *Streptococcus faecium* [5], and *Escherichia coli* RT500 [6]. Convincing evidence which showed that the drastic increases of pK_a for TMP is the result of hydrogen bonding between N-1 and the carboxyl group of the aspartic residue at the active site was provided by a ^{13}C NMR study of [2-^{13}C]TMP in complex with two *E. coli* mutant enzymes in which Asp-27 is replaced by asparagine and by serine, respectively [7]. While the pK_a of TMP in complex with the wild-type enzyme is elevated from 7.6 in free TMP to above 10, the pK_a of the inhibitor bound to the mutant enzymes is lowered to a value below 4.0. NMR results further showed that the ternary complex of TMP with *L. casei* DHFR and $NADP^+$ exists in two conformations [8]. DHFR from *E. coli* and *L. casei* have an amino acid sequence homology of less than 30%. However, X-ray diffraction investigations revealed that the overall backbone geometries of the two bacterial reductases are almost identical [2]. In contrast, NMR studies detected two conformations for the binary complex of *L. casei* DHFR with folate [8], while only a single conformation is observed for the *E. coli* DHFR/folate complex [9]. It is therefore of interest to compare the conformational aspects of these two species in other complexes. In this letter we report our initial results of ^{15}N and ^{31}P NMR studies of the conformations of TMP bound to *E. coli* wild-type DHFR.

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2. MATERIAL AND METHODS

Wild-type *E. coli* DHFR was isolated from an DHFR overproducing *E. coli* strain carrying multiple copies of the pUC8 plasmid (a generous gift of Dr. E. Howell of the University of Tennessee). DHFR was isolated and assayed according to Baccanari et al. [10]. Enzyme purity was checked with denaturating SDS-gel electrophoresis. Protein concentration was determined by the method of Bradford using Pierce protein assay reagent (Pierce Chemical Co.) [11].

The samples used for solution NMR studies contained 1.5–2.0 mM of enzyme in 50 mM phosphate (^{15}N NMR) or 10 mM Tris-HCl (^{31}P NMR) buffer, 100 mM KCl and 1 mM EDTA in D_2O. Specifically labelled TMP was dissolved in DMSO and added in appropriate amounts to the enzyme solution in microliter quantities. Values of pH were direct readings (without correction for deuterium isotope effect)

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from an Orion model 811 pH meter equipped with an Ingold 6030-2 electrode. All the solution NMR spectra were obtained with a Varian XL-400 spectrometer operating at 161.892 MHz for ^{31}P and 40.547 MHz for ^{15}N. Experimental conditions were: single pulse (30° flipping angle) experiment with WALTZ-16 decoupling; spectral width = 5000 Hz (^{31}P) or 8110 Hz (^{15}N) at 16k data point resolution. Recycle time = 1.8 s (^{31}P) and 1.2 s (^{15}N). Spectra were obtained at 23°C. ^{15}N chemical shifts were referenced to external 0.1 M $^{15}NH_4Cl$ in 0.1 N HCl. ^{31}P chemical shifts were referenced to external phosphate sample at pH = 8.0.

3. RESULTS AND DISCUSSION

Fig. 1 shows five proton-decoupled solution ^{15}N NMR spectra of [1,3,2-amino-$^{15}N_3$]TMP in various protein complexes: (i) TMP/DHFR, pH = 6.8; (ii) TMP/NADPH/DHFR, pH = 7.3; (iii) TMP/NADP$^+$/DHFR, pH = 6.3; (iv) TMP/NADP$^+$/DHFR, pH = 7.4; and (v) TMP/NADP$^+$/DHFR, pH = 8.4. The resonances due to free TMP can be identified from their shift with pH and are marked with a ×. The other resonances can be readily assigned to the three nitrogens based on their chemical shifts as determined above and assigned previously [12]. The chemical shift of the N-1 resonance is very close to that of the protonated form and is nearly invariant in the pH range of 6.4–8.4, suggesting that the pK_a of bound TMP is much higher than 8.4 and N-1 is the protonation site, similar to that found in the *L. casei* DHFR complex [12]. The increased pK_a of TMP bound to *L. casei* DHFR has been interpreted to be due to the interaction between N-1 nitrogen and the carboxyl of Asp-26. Therefore, our results suggest that such an interaction is retained when TMP is bound to *E. coli* DHFR, presumably between N-1 and the carboxyl of Asp-27.

For the TMP/DHFR binary complex (Fig. 1a) three resonances due to the three nitrogens were observed, suggesting that TMP is bound in a single conformation in the binary complex. Similarly a single conformation was observed for the ternary complex of TMP/NADPH/DHFR (Fig. 1b). For the ternary complex of TMP/NADP$^+$/DHFR (Fig. 1c–e) the ^{15}N NMR spectrum clearly showed the presence of two resonances for each of the three nitrogens at their respective chemical shift regions. The chemical shifts of these resonances exhibit only slight variation with pH. Thus, the ternary complex in solution can be assigned to two conformations, designated as I_1 and I_2. The relative population of a given conformation can be determined by dividing the intensity of each resonance by the total intensity of a given nitrogen. From the intensity variations with pH one can readily assign a given resonance to a particular conformation. Thus at pH 7.4 the resonances at 63.3, 113.2 and 180.0 ppm are assigned to conformation I_1 and those at 59.3, 111.5 and 179.0 ppm are assigned to conformation I_2. The variation with pH of conformer I_1 is shown on Fig. 3. The largest difference in chemical shift between the two conformations occurs at the 2-amino nitrogen (4 ppm) while the N_3 nitrogen experiences the least (1 ppm). The large chemical shift difference of 4 ppm at the 2-amino nitrogen is hard to be reconciled on the basis of ring current shift alone and must contain contributions from other sources such as strength of hydrogen bonding. From the linewidths of these resonances the rate of interconversion between these two conformers are estimated to be less than 5 s^{-1}.

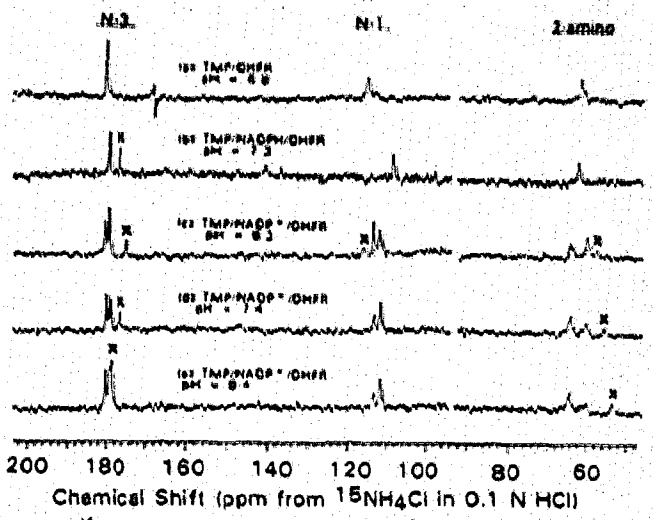

Fig. 1. ^{15}N NMR spectra of [1,3,2-amino-$^{15}N_3$]TMP in complex with DHFR in the absence and presence of cofactors, NADP$^+$ and NADPH. NMR samples contain 1 mM DHFR in 50 mM phosphate buffer, 100 mM KCl, 1 mM EDTA in D_2O. Spectra were taken at 22°C.

Fig. 2 shows a set of ^{31}P NMR spectra of various cofactor/DHFR complexes. In the dianionic form the 2′-phosphate resonates at between 1 and 3 ppm and the pyrophosphates resonate at between −11 and −17 ppm [13]. The ^{31}P NMR spectra of the binary complexes of NADP$^+$/DHFR (Fig. 2a) and NADPH/DHFR (Fig. 2b) consist of three resonances, corresponding to the 2′-phosphate and the two non-equivalent

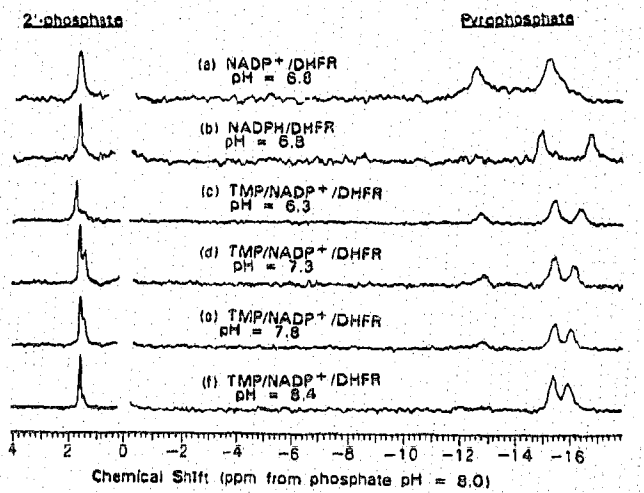

Fig. 2. ^{31}P NMR spectra of NADP$^+$ and NADPH bound to *E. coli* DHFR in the presence and absence of TMP. Samples contain 1 mM DHFR in 10 mM Tris buffer, 100 mM KCl, 1 mM EDTA in D_2O. Spectra were taken at 10°C.

pyrophosphate resonance. The 2'-phosphates resonate at similar frequency at 1.69 and 1.67 ppm for $NADP^+$ and NADPH, respectively. However, the pyrophosphates of the two complexes are very different: −12.5 and −15.3 ppm for $NADP^+$ and −15.0 and −16.9 ppm for NADPH. Thus the conformation of the pyrophosphate of the two complexes are different. At pH between 6.3 and 8.4 (Fig. 2c-f), two resonances for the 2'-phosphate and three resonances for the pyrophosphate were observed for the ternary complex of TMP/$NADP^+$/DHFR. From the intensities of these resonances, obtained by integration for the pyrophosphate resonances or by deconvolution for the 2'-phosphate resonances, we can readily assign them to two conformations, designated as C_1 (1.69, −15.4, and −16.1 ppm) and C_2 (1.49, −12.9, and −15.4 ppm). The variation with pH of the C_1 conformer population is shown on Fig. 3. This assignment is in agreement with that of Birdsall et al. [14]. According to this assignment, NADPH is in conformation C_1 in both the binary and ternary complexes while $NADP^+$ is in conformation C_2 in the binary complex and exists in a mixture of C_1 and C_2 conformations in the ternary complex. At low pH the two conformers exist in roughly equal populations. Raising the pH favors conformation C_1. This ^{31}P MNR pattern is very similar to that observed in the ternary complex of TMP and $NADP^+$ with *L. casei* DHFR [14]. A major difference between the two enzymes is the observation of two resonances for the 2'-phosphate in the *E. coli* enzyme complex while only a single resonance was detected for the *L. casei* complex.

Since both TMP and $NADP^+$ exist in two conformations in the ternary complex of TMP/$NADP^+$/DHFR the protein complex can potentially exist in two to four conformations, depending on whether the conformations at TMP and $NADP^+$ binding sites are correlated. We have investigated this question by examining the change with pH of the relative populations of the conformers (Fig. 3). The data points presented in Fig. 3 are the averaged integrated (or convoluted) intensities of I_1 (63.3, 113.2, and 180.0 ppm resonances on the ^{15}N spectra) and C_1 (1.69 and −15.4 ppm resonances on the ^{31}P spectra) resonances as described above. For TMP, conformation I_1 increases from 42 ± 5% at pH 6.3 to 63 ± 5% at pH 8.4. Whithin the same pH range, conformation C_1 increases from 57 ± 5% to 88 ± 8%. Furthermore, the change in the distribution of the two conformations occurs at lower pH range for the TMP moiety and at higher pH range for the $NADP^+$ moiety. Consequently, even though the distribution of the two conformations for the two ligands are similar at neutral pH, they are quite different at pH 6.3 and 8.4. Thus it appears that the conformations at the TMP and the $NADP^+$ binding sites may be affected by different groups with substantially different pK_a. The implication of this observation is that the protein ternary complex may be able to adopt up to four conformations. The relative populations of these conformations are pH-dependent. We should point out that significant changes in NMR chemical shift may result from small changes in atomic position. Additional experiments are in progress for characterizing these conformations.

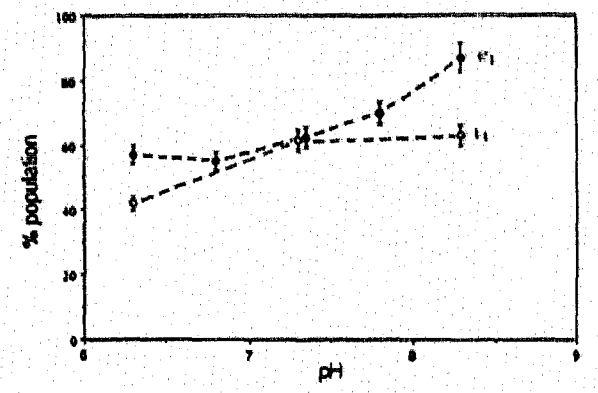

Fig. 3. Variation with pH of the populations of conformations T_1 (open circle) and C_1 (filled circle)

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