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Methotrexate binds in a non-productive orientation to human dihydrofolate reductase in solution, based on NMR spectroscopy

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Dihydrofolate reductase (DHFR) is an intracellular target enzyme for folate antagonist drugs, including methotrexate. In order to compare the binding of methotrexate to human DHFR in solution with that observed in the crystalline state, NMR spectroscopy has been used to determine the conformation of the drug bound to human DHFR in solution. In agreement with what has been observed in the crystalline state, NOE's identified protein and methotrexate protons indicate that methotrexate binds in a non-productive orientation. In contrast to what has been reported for *E. coli* DHFR in solution, only one bound conformation of methotrexate is observed.

Dihydrofolate reductase; Protein NMR; Protein-drug interaction; Methotrexate

1. INTRODUCTION

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Tetrahydrofolate or its derivatives are essential co-factors in the biosynthesis of purine nucleotides, thymidylate and several amino acids. Failure to maintain adequate levels of tetrahydrofolate reduces cellular thymidylate and purine levels, resulting in decreased nucleic acid synthesis [1]. Based on this metabolic consequence, DHFR is a pharmacologically important intracellular target enzyme for a number of folate antogonists, including the anticancer drug methotrexate and the antibacterial agent trimethoprim. Recently, pneumocystis DHFR has become an important anti-folate target since pneumocystis infection is the main cause of death in patients with acquired immunodeficiency syndrome [2].

Crystallographic studies of human DHFR indicate that substrate folate and inhibitor methotrexate bind in different conformations [3,4]. Whereas folate binds in a stereochemically correct orientation for reduction, methotrexate binds in a non-productive orientation with the pteridine ring rotated by 180° about the C6-C9 bond. This observation is consistent with structural studies of DHFR from bacterial [5–9] and other mammalian [10] sources. The question as to whether or not methotrexate binds precisely the same in an enzyme complex in the crystalline vs the solution state has been addressed. Solution NMR spectroscopy studies reported herein confirm that methotrexate binds to human DHFR in a single non-productive orientation, similar to that in the crystalline state.

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Abbreviations: DHFR, dihydrofolate reductase; DQF-COSY, double quantum filtered correlated spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy

2. MATERIALS AND METHODS

Recombinant human DHFR was prepared as previously described [11]. Samples for NMR spectroscopy typically contained 1–2 mM protein and 25 mM KCl in 50 mM phosphate buffer at pH 6.5. Samples dissolved in ^2H_2O were prepared by repeatedly concentrating the solution under a stream of nitrogen gas and adding ^2H_2O. Phase-sensitive NOESY [12] and DQF-COSY [13] spectra were recorded on a GN-500 spectrometer at 298K. Data were processed using the program FELIX from Hare Research, Inc. Proton chemical shifts were referenced to the ^1HDO signal at 4.76 ppm.

3. RESULTS

Resonances of bound methotrexate protons were distinguished from those of the protein and assigned using the following strategy. In Fig. 1, NOE's are observed correlating to a resonance at 7.91 ppm. The relative sharpness of this resonance suggests that it is not scalar coupled to other protons. That this resonance is not from the polypeptide is proven by the fact that it does not give rise to a ^1H–^{13}C or ^1H–^{15}N cross peak in heteronuclear correlation spectra of $^{13}C/^{15}N$ doubly-labeled polypeptide complexed with unlabeled

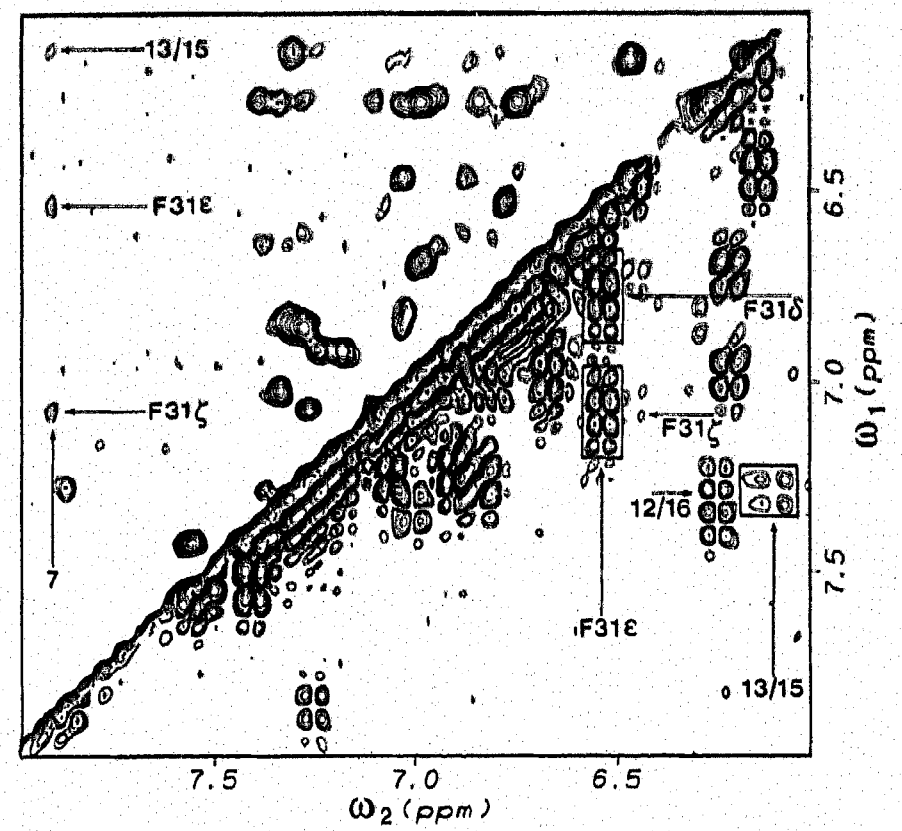

Fig. 1. Regions of the 100 ms NOESY (above the diagonal) and DQF-COSY (below the diagonal) spectra of human DHFR in 100% ^2H_2O. NOE's to the methotrexate H7 resonance are indicated.

methotrexate (data not shown). This resonance is thus assigned to H7 of the pteridine ring of methotrexate. The benzoyl moiety of methotrexate is manifested in the DQF-COSY spectrum by a correlation between the H12/16 (7.33 ppm) and H13/15 (6.15 ppm) protons. They are distinguished by virtue of an NOE between the H7 and H13/15 protons, as indicated in Fig. 1. As shown in Fig. 2, both the H7 and H13/15 protons have an NOE to a resonance at 3.39 ppm, which is thereby assigned to the methyl group on N10. The N10 methyl group has additional NOE's to resonances at 4.48 and 5.11 ppm (not shown), which are assigned to the methotrexate H9 protons.

Fig. 1 indicates two other aromatic NOE's to the methotrexate H7 resonance. These two resonances arise from the ε- and ζ-protons of a phenylalanine spin system, delineated in the lower part of Fig. 1. In Fig. 2, NOE's are observed from the H7 proton to two resonances at high field. Heternonuclear three-dimensional NMR spectroscopy [14,15] of uniformly ^{15}N-labeled human DHFR has permitted assignment of more than 90% of the polypeptide (unpublished

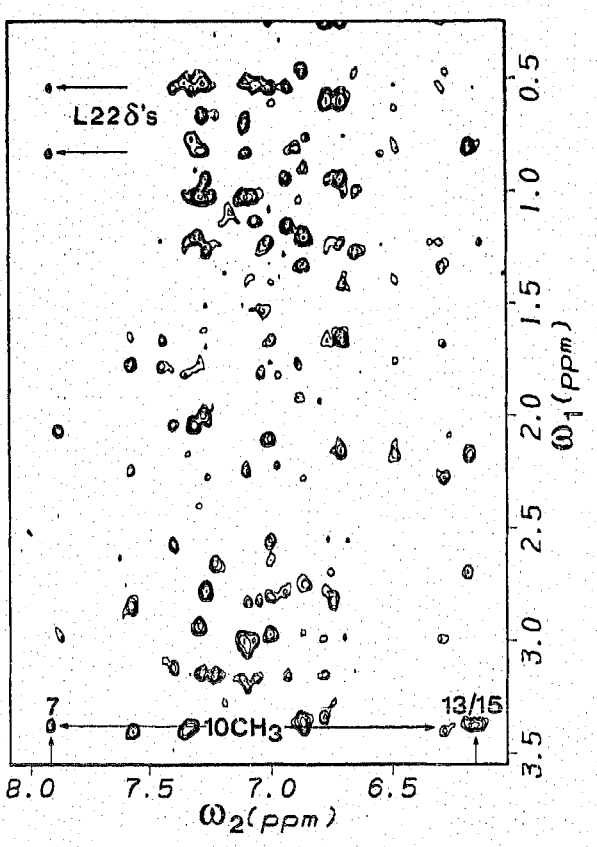

Fig. 2. Region of the 100 ms NOESY spectrum of human DHFR showing interactions from the methotrexate H7 and H13/15 protons to aliphatic protein and methotrexate protons.

results). Using these results, the phenylalanine spin system in Fig. 1 is that of phenylalanine-31. Likewise, the high field resonances in Fig. 2 arise from the two δ-methyl groups of leucine-22.

4. DISCUSSION

Observation of NOE's from the H7 proton to the side chains of leucine-22 and phenylalanine-31 indicate that methotrexate binds in a non-productive orientation, analogous to what is observed in the crystalline state [3]. These NOE's would not be present if the pteridine ring was bound in the folate-like orientation. In addition, the pattern of NOE's observed among the set of methotrexate protons indicates that the conformation of the drug when bound to human DHFR in solution is similar to that in the X-ray crystal structure [3].

In contrast to what is detected by solution NMR spectroscopy of *E. coli* DHFR [9], only one set of resonances for bound methotrexate was observed under the solution conditions used. If more than one binding conformation existed, a second set of methotrexate resonances giving rise to slightly different NOE's to nearby amino acid side chains would have been observed. Clearly, only one set of NOE's is observed to the leucine-22 and phenylalanine-31 side chains in Figs. 1 and 2. However, different conformers in fast exchange cannot be ruled out, since this would give rise to only one set of methotrexate resonances. Interestingly, some proton resonances of the protein display some heterogeneity, which may indicate the existence of two or more protein isomers. An example of this occurs for the amide protons of residues near and including leucine-22. Since asparagine-19 and several prolines (residues 23, 25 and 26) are located in this region, asparagine deamidation or *cis-trans* proline isomerization may be responsible for these observations. These possibilities are currently being investigated.

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