

# Direct $^{19}\text{F}$ NMR observation of the conformational selection of optically active rotamers of the antifolate compound fluoronitropyrimethamine bound to the enzyme dihydrofolate reductase

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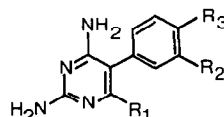
The molecular basis of the binding of the lipophilic antifolate compound fluoronitropyrimethamine [2,4-diamino-5-(4-fluoro-3-nitrophenyl)-6-ethylpyrimidine] to its target enzyme dihydrofolate reductase has been investigated using a combination of  $^{19}\text{F}$  NMR spectroscopy and molecular mechanical calculations.  $^{19}\text{F}$  NMR reveals the presence of two different conformational states for the fluoronitropyrimethamine-*Lactobacillus casei* enzyme complex. MM2 molecular mechanical calculations predict restricted rotation about the C5-C1' bond of the ligand and this gives rise to two slowly interconverting rotamers which are an enantiomeric pair. The results of  $^{19}\text{F}$  NMR spectroscopy reveal that both these isomers bind to the enzyme, with different affinities. There is no detectable interconversion of the bound rotamers themselves on the NMR timescale. The effect of the addition of co-enzyme to the sample is to reverse the preference the enzyme has for each rotamer.

Dihydrofolate reductase;  $^{19}\text{F}$ -NMR; Pyrimethamine; Antifolate; Conformational selection

## 1. INTRODUCTION

Lipophilic antifolates based on the antimalarial agent pyrimethamine **1** (scheme 1) have been developed to treat tumours insensitive to the anti-neoplastic agent methotrexate due to an inhibition of the active uptake of the drug into cells [1]. We have been investigating the molecular basis of the binding of a compound of this class, fluoronitropyrimethamine [2] [2,4-diamino-5-(4-fluoro-3-nitrophenyl)-6-ethylpyrimidine] **2**, to its target enzyme dihydrofolate reductase. By analogy with *o*-substituted biphenyls restricted rotation about the

C5-C1' bond of the compound would be expected to give rise to two slowly interconverting rotamers which are an enantiomeric pair. Here we explore



|   | R <sub>1</sub>                | R <sub>2</sub>  | R <sub>3</sub> |
|---|-------------------------------|-----------------|----------------|
| 1 | C <sub>2</sub> H <sub>5</sub> | H               | Cl             |
| 2 | C <sub>2</sub> H <sub>5</sub> | NO <sub>2</sub> | F              |
| 3 | CH <sub>3</sub>               | Cl              | Cl             |
| 4 | C <sub>2</sub> H <sub>5</sub> | N <sub>3</sub>  | Cl             |

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the potential of NMR spectroscopy for detecting the bound states of the different enantiomers.

## 2. MATERIALS AND METHODS

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R [3]. Fluoronitroprimethamine was synthesised by the method previously described [2]. 376 MHz  $^{19}\text{F}$  spectra were obtained using a Bruker AM-400 spectrometer. The 0.4 ml samples contained 1.2 mM enzyme, 50 mM potassium phosphate, pH\* 6.5, 500 mM potassium chloride, 1 mM EDTA, 1 mM dioxan and excess fluoronitroprimethamine in  $^2\text{H}_2\text{O}$  (pH\* denotes a meter reading uncorrected for the deuterium isotope effect on the glass electrode). The two-dimensional exchange experiment was carried out in the phase-sensitive mode using the NOESY pulse sequence [4]. 1024 data points were recorded in  $t_2$  for each of 64  $t_1$  values.

## 3 RESULTS AND DISCUSSION

The  $^{19}\text{F}$  spectrum of the fluoronitroprimethamine-*Lactobacillus casei* enzyme complex has two signals (see fig.1a). The additional intense peak referenced at 0 ppm is the resonance from free ligand. The signals marked A and B are due to bound compound which appears to be in slow exchange with the free ligand. They represent different conformations of the ligand-enzyme complex. The populations of these two conformations are different with the ratio of conformations A:B being 0.6:0.4.

Restricted rotation in *o*-substituted biphenyls is well known [5] and would also be expected in *o*-substituted phenyl-pyrimidines. Molecular mechanical calculations using the MM2 package [6] with modified Allingers force field constants [7] indicate that for free fluoronitroprimethamine the rotation about the diaminopyrimidine-5-phenyl ring bond will be severely restricted, the highest energy conformation being that with the rings coplanar as shown in fig.2. Thus the ligand will normally exist as two rotamers, with the nitro group either above or below the plane of the diaminopyrimidine ring. In NMR experiments on the analogous compound 2,2'-bis(acetoxymethyl)-diphenyl, Meyer and Meyer [5] have detected separate rotamers and have estimated the rate of rotation to be  $0.25\text{ s}^{-1}$  at  $94^\circ\text{C}$ . We suggest that the two  $^{19}\text{F}$  signals A and B correspond to two such rotamers bound to the enzyme.

Fig.1b shows a two-dimensional  $^{19}\text{F}$  NOESY/exchange experiment where the off diagonal cross

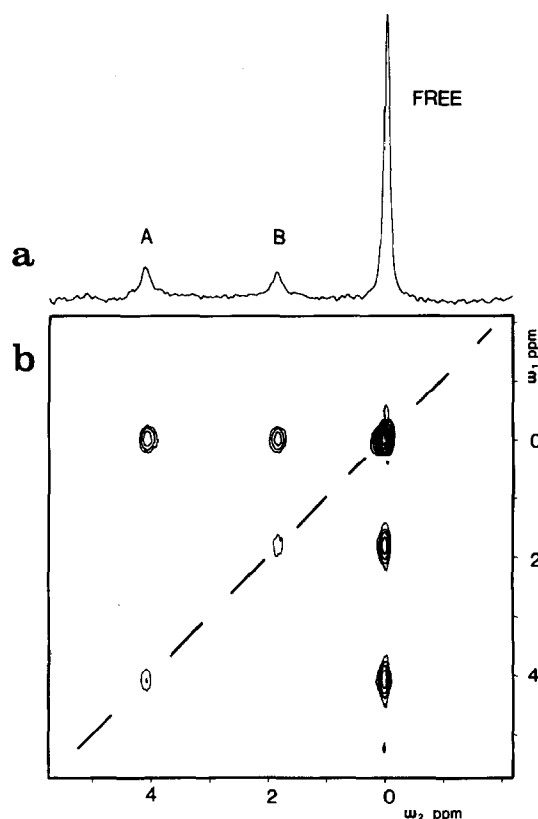


Fig.1. 376 MHz  $^{19}\text{F}$  spectra of the dihydrofolate reductase-fluoronitroprimethamine complex recorded at 308 K. (a) One-dimensional spectrum; (b) 2-dimensional NOESY/exchange spectra. Both spectra are referenced to the free ligand signal.

peaks indicate a transfer of magnetisation from one site to another. While cross peaks are observed between the signals for the free compound and those for both conformation A and conformation B, no cross peaks are observed between signals A and B thus indicating that there is no detectable exchange between the two conformations themselves on the NMR timescale. This is in agreement with our model that conformers A and B arise from the binding of the two non-equivalent rotamers shown in fig.2.

The effect of adding increasing amounts of  $\text{NADP}^+$  to the sample is shown in fig.3. The coenzyme causes a marked perturbation of the signals A and B and two new resonances are seen. NOESY/exchange experiments with a fluoronitroprimethamine-enzyme complex partially covered with  $\text{NADP}^+$  (not shown) indicate that signal B exchanges sufficiently rapidly with B' to give rise to a

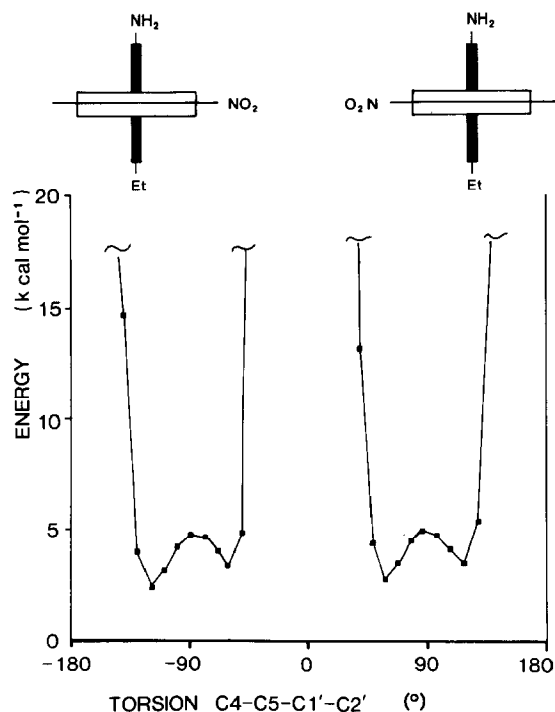


Fig.2. MM2 molecular mechanical calculation of the potential energy dependence as a function of 36  $10^\circ$  rotations about the torsion angle C4-C5-C1'-C2' of the fluoronitropyrimethamine structure.

cross peak. This indicates that peak B becomes B' (and thus A becomes A') on addition of coenzyme. It is seen from fig.3 that the addition of coenzyme not only changes the environment of the fluorine nucleus in each of the rotamers, but also changes the populations of each rotamer bound to the enzyme. The ratio of A:B changes from 0.6:0.4 in the binary complex to 0.3:0.7 in the ternary complex, indicating that the effect of the coenzyme is to reverse the preference the enzyme has for binding to each of the rotamers.

For a normal racemic mixture of optical isomers, the isomers can be resolved thus allowing the individual binding constants to be obtained for the individual components. In the case of a mixture of optical isomers resulting from rotamers which can interconvert slowly this provides an effectively irresolvable mixture which cannot be examined by this method. Here we show that NMR spectroscopy can provide a direct method of determining the ratios of the binding constants of the two isomers present as an interconverting mixture.

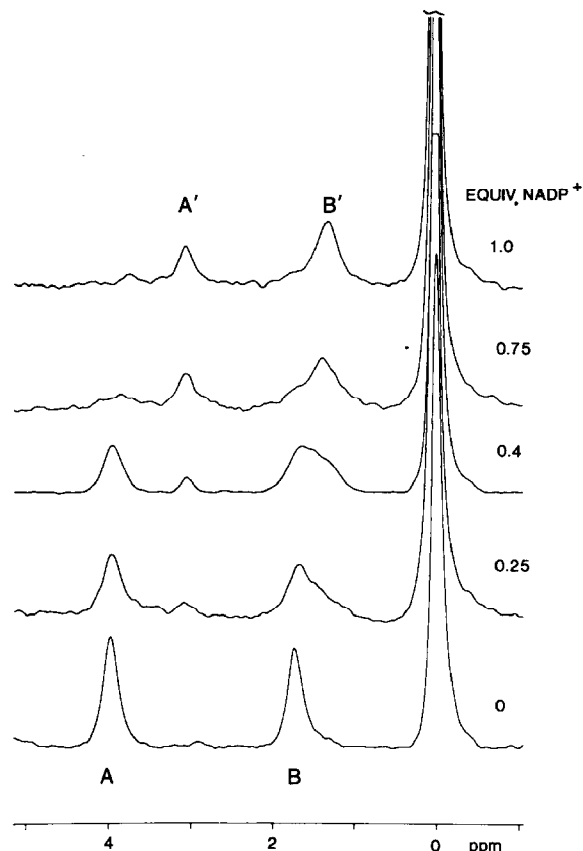


Fig.3. The effect of the addition of increasing saturation of  $\text{NADP}^+$  to the dihydrofolate reductase-fluoronitropyrimethamine complex. Spectra were recorded at 308 K.

We have previously shown that of the two rotameric forms of folinic acid (from hindered rotation about the bond between N5 and the formyl group) only one is bound to dihydrofolate reductase [8]. We now show that for a ligand with restricted rotation which exists as non-equivalent rotamers undergoing slow interconversion, both rotamers may bind to the target site thus interacting with different groups on the protein. Likewise each rotamer may have different affinities for the target, and these affinities for both can be perturbed by the binding of a second ligand. The lipophilic antifolate compounds metoprin [9] 3 and *meta*-azidopyrimethamine [2] 4 have similar chemical structures and would be expected to show the same phenomenon. It may also be the case that different rotamers may bind to different biological targets in the same way as the optical isomers of drugs such as d-ketamine [10] and barbiturates [11] bind to

different sites. Chemical modification to prevent the interconversion of rotamers allowing the synthesis of single rotamers may be used in the development of more selective agents.

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