THE USE OF SATURATION TRANSFER NMR EXPERIMENTS TO MONITOR THE CONFORMATIONAL SELECTION ACCOMPANYING LIGAND–PROTEIN INTERACTIONS

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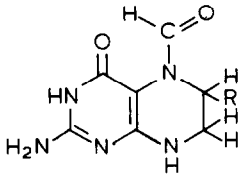
1. Introduction

A flexible molecule in free solution exists as a mixture of interconverting conformers, but when such a molecule binds to a protein it commonly adopts a unique conformation. In this case ligand binding involves a process of conformational selection, which must influence the thermodynamics and kinetics of the overall binding reaction [1,2]. The conformations of bound ligands have been studied quite extensively, principally by X-ray crystallography but also in solution by NMR spectroscopy [3–6]. We show here that, in suitable circumstances, NMR can be used not only to determine the conformation of a ligand in its complex with a protein but also to obtain information on the conformational selection process itself.

Folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid) is a stable analogue of the product, tetrahydrofolate, of the reaction catalysed by dihydrofolate reductase and it binds tightly ($K_a = 10^8\ M^{-1}$) to the enzyme from *Lactobacillus casei* (B. B., A. S. V. B., unpublished). In solution, folinic acid exists as a mixture of the two rotameric forms I and II: (where R = $-CH_2NH-C_6H_4-CO-$L-Glu). The interconversion of forms I and II is relatively slow, so that separate ^1H NMR spectra are observed. Fig.1a shows the two formyl proton signals of free folinic acid, the more populated form I having its formyl proton oriented towards the



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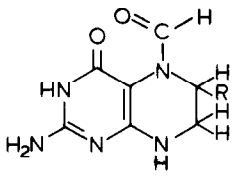

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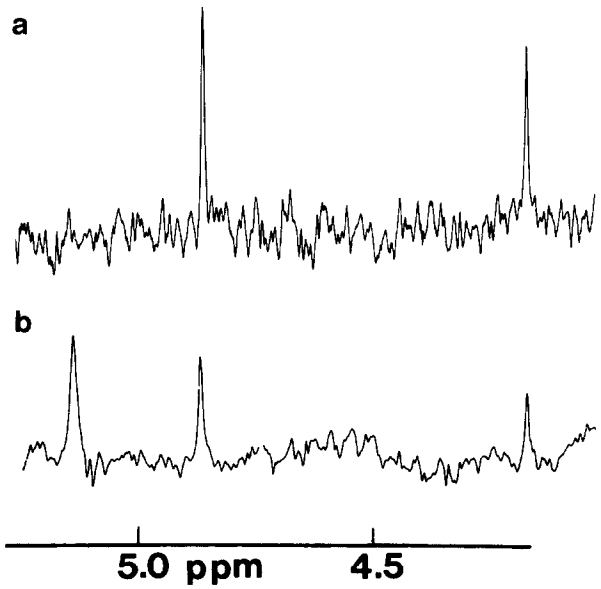

Fig.1. The low field region of the 270 MHz ^1H spectrum showing the formyl proton signals of (a) (6*RS*,α*S*)-folinic acid alone (b) 2 equivalents of (6*RS*,α*S*)-folinic acid (2 mM) in the presence of 1 equivalent of dihydrofolate reductase. (1 mM). A selectively deuterated sample of dihydrofolate reductase with all its aromatic protons except the Tyr H2′ and H6′ protons replaced by deuterium was used in this experiment.

4-oxo group of the pteridine ring [7]. We show here how transfer of saturation NMR experiments can be used to determine which rotameric form of folinic acid binds predominantly to dihydrofolate reductase.

2. Materials and methods

(6*RS*,α*S*)-folinic acid was obtained from Sigma Chemicals and ^2H_2O (99.85 atom% ^2H) from Norsk

Hydroelectrisk. The preparation and purification of the normal and selectively deuterated dihydrofolate reductase from *Lactobacillus casei* MTX/R were done as in [8,9]. The purified enzyme was lyophilised twice from ^2H_2O solution to remove most of the exchangeable protons and then redissolved to give 1 mM enzyme solutions, in ^2H_2O containing 50 mM potassium phosphate and 500 mM KCl at pH* 6.9 (uncorrected meter reading: meter calibrated with buffers in H_2O solution). The folinic acid was added to the enzyme solutions as μl volumes of a 28 mM stock solution.

The ^1H NMR spectra were obtained at 270 MHz using a Bruker WH270 spectrometer operating in the Fourier transform mode as in [4]. The transfer of saturation experiments [10] were carried out using the ^1H decoupler to provide the second irradiating frequency. The pulse sequence used in these experiments was $(t - \tau - \pi/2 - AT - T)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t (0.8–1.0 s) and where τ is a short delay (2 ms) to allow for electronic recovery after the selective irradiation is removed. The acquisition time AT was usually 0.975 s and an additional delay T of 3.0 s allowed for most of the magnetisation to recover before repeating the sequence [4,11].

3. Results and discussion

Commercial folinic acid is a mixture of two diastereoisomers; both have the *S*-configuration at the α-carbon of the glutamate moiety, but they differ in the configuration at C6 of the pteridine ring. The two diastereoisomers have identical ^1H NMR spectra. The mixture can be used successfully in studies of folinic acid binding to *L. casei* dihydrofolate reductase because the natural (6*S*,α*S*)-diastereoisomer binds $\geqslant$1000-times more tightly than the (6*R*,α*S*)-compound ($K_a = 10^8\ M^{-1}$ for (6*S*,α*S*)-folinic acid; B. B., A. S. V. B., unpublished).

Therefore, a solution containing 1 mM dihydrofolate reductase and 2 mM (6*RS*,α*S*)-folinic acid will effectively contain only the (6*S*,α*S*)-folinic acid–enzyme complex and free (6*R*,α*S*)-folinic acid. The low field region of the ^1H spectrum of such a solution is shown in fig.1b. In addition to the formyl proton resonances at 4.16 and 4.88 ppm from free (6*R*,α*S*)-folinic acid, a third resonance is observed at 5.19 ppm which must arise from the formyl proton of bound (6*S*,α*S*)-folinic acid; this assignment is confirmed by the transfer of saturation experiments below. The intensity of this formyl proton resonance from the bound ligand corresponds to one proton, i.e., it represents, within experimental error, all the bound ligand. This demonstrates that in the bound state the formyl group of (6*S*,α*S*)-folinic acid adopts predominantly a single conformation.

The process of conformational selection between forms I and II can be represented by

where E.I and E.II represent the complexes of the enzyme with forms I and II, respectively, of folinic acid. We have used transfer of saturation experiments to establish whether E.I or E.II predominates, and to throw light on the kinetic pathway of the conformational selection process.

Fig.2 shows that irradiation at the resonance position of the formyl proton of bound (6*S*,α*S*)-folinic acid (5.19 ppm) leads to a substantial decrease in the intensity of the signal from free form I, but not that from free form II folinic acid. (The sample used for the experiment shown in fig.2 contained 5 mM (6*RS*,α*S*)-folinic acid, and 1 mM enzyme, so that 38% of the intensity of the 'free' signals arises from (6*S*,α*S*)-folinic acid which is exchanging with the enzyme complex; the remaining 62% arises from (6*R*,α*S*)-folinic acid which is not interacting with the enzyme under these conditions.)

This decrease in intensity of the 'free' signal arises from chemical exchange of the folinic acid molecules between the bound state, where the formyl proton is 'saturated', and the free state. This decrease in intensity indicates that the rate of exchange of the folinic acid between these two states is at least of the same order as the spin-lattice relaxation rate of the formyl proton in the free molecule. The absence of an intensity change on the signal of free form II implies that the exchange rate between free forms I and II is low compared with the relaxation rate of form II.

An exhaustive search over a wide range of irradiation frequencies failed to reveal any other position at which transfer of saturation effects on either of the

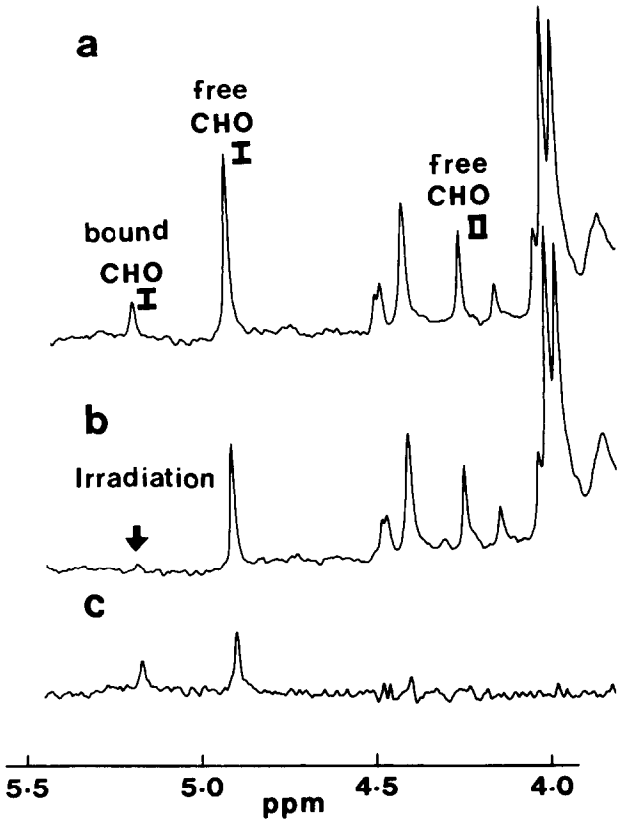

Fig.2. The low field region of the 270 MHz ^{1}H spectrum of 5 mM (6*RS*,α*S*)-folinic acid in the presence of 1 mM dihydrofolate reductase at pH 6 and 45°C (a) with irradiation outside the spectral region, (b) with irradiation at 5.19 ppm (c) difference spectrum between (a) and (b) indicating the change in intensity at the position of free CHO (I).

free signals could be observed. This shows that the bound folinic acid molecule exchanges at a detectable rate only with form I of free folinic acid, not with form II. These observations can most simply be explained by postulating that:

(a) The conformation of the formyl group of bound (6*S*,α*S*)-folinic acid corresponds to form I above - i.e. with the formyl proton oriented towards the 4-oxo group; and

(b) The conformational selection process can be simply represented by:

$$E.I \rightleftharpoons I \rightleftharpoons II$$

The existence of a small proportion (<5%) of the E.II complex can not be ruled out. However, the transfer of saturation experiment shows that binding of form II followed by isomerisation on the enzyme is not a major route for the formation of the favoured E.I complex. Direct binding of form I of folinic acid is the only kinetically significant pathway.

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