STRUCTURAL INVESTIGATION OF FOLIC ACID BY NMR PROTON RELAXATION AND MOLECULAR MECHANICS ANALYSIS

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1 Introduction

Folic acid N-[4-(2-Amino-4-hydroxy-pteridinyl-(6)-methylamino)-benzoyl]-L-aminoglutaric acid (Figure 1) is a fundamental coenzyme involved in one-carbon unit transfer processes[1]. The solid state conformation of folic acid has been defined but there have been few investigations on the structure of this coenzyme in solution[2].

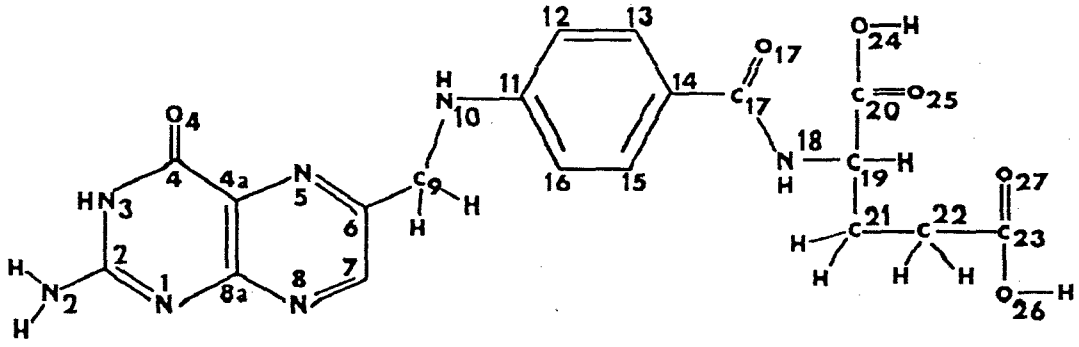

Figure 1: Structure and numbering of folic acid.

Its role is also fundamental in reductive enzymatic processes in which tetrahydrofolate (the reduced form of folic acid) is oxidized to dihydrofolate and folate. The dihydrofolate reductase NADPH-dependent enzyme controls the biological level of tetrahydrofolate[3].

In the present paper the conformational properties of this molecule were analyzed by Nuclear Magnetic Resonance (NMR). Information on dynamical motion, magnetic dipolar connectivities and energy minimization calculations are combined in order to define the solution structure of folic acid.

2 Experimental

Two-dimensional COSY, NOESY and Hetcor experiments were obtained by $(\pi/2\text{-}t_1\text{-}\pi/2\text{-}AT)_n$,[4] $(\pi/2\text{-}t_1\text{-}\pi/2\text{-}t_m\text{-}\pi/2AT)_n$[5] and $[(\pi/2)_H\text{-}t_1/2\text{-}\pi_C\text{-}t_1/2\text{-}(\pi/2)_H(\pi/2)_C\text{-}AT]$[6] pulse sequences respectively. Spin-lattice relaxation rates were measured using the $(180°\text{-}\tau\text{-}90°\text{-}t)_n$ pulse sequence. The NMR measurements were performed using a 0.12 mol.dm^{-3} DMSO-d_6 solution at 27°C. NMR spectra were recorded on a Varian XL-200

and a Bruker AMX-600 spectrometers operating at 200 and 600 MHz respectively. Molecular mechanics calculations were computed by the MacroModel program, version 2.5[7] implemented on a Vax 11/750 computer. The force field used was that reported by Weiner et al[8].

3 Results and Discussion

In the present paper the NMR properties of folic acid were investigated in depth in order to define the molecular structure in DMSO-d_6 solution.

As the proton and carbon assignments of folic acid refer only to water solution at basic pH[9,10], both proton and carbon chemical shifts in DMSO-d_6 were determined by a method based on conventional two-dimensional COSY and Hetcor experiments. Figures 2 and 3 show the COSY and Hetcor spectra of folic acid. The complete proton assignments and chemical shifts of protonated carbons can be determined from these sets of data. The quaternary carbons were assigned by a frequency-dependent selective proton-carbon NOE experiment[11,12].

The results obtained are reported in Table 1. The strategy for structural analysis was based on a combined approach. First we studied the dynamical properties of folic acid in solution which led to two possible scenarios:

i) the molecular motion is subject to different degrees of freedom, in which case each molecular moiety behaves independently as a consequence of the lack of "ordered" elements;

ii) the molecule is subject to overall dynamical reorientation, characterized by a single rotational correlation time, τ_c.

These conditions, for molecules of the size of folic acid, are verified whenever non-covalent interactions stabilize specific conformations.

The appropriate method for dynamical investigation is based on analysis of the carbon spin-lattice relaxation rate, R_{1C}. The experimental R_{1C}, calculated for protonated carbons, are reported in Table 1. From these data and using the Allerhand's approach[13] a unique correlation time value of 3×10^{-10} s was calculated. In Table 1 the selective and non-selective proton relaxation rates are also reported. These experimental values confirm the dynamical region of the isotropic molecular motion of folic acid in solution. A second set of structural information can be derived from the study of the extent of the dipolar magnetization transfer in the protonic environment.

In this case the NOESY spectrum can provide the complete network of the proton-proton dipolar interactions, which is related to internuclear distances. The analysis of the NOESY spectrum enables us to identify proton pairs in which the cross-relaxation contribution is significant. These include the NH(10)-H(12/16), NH(10)-H(9), H(12/16)-H(9), H(12/16)-H(13/15) and H(18)-H(21b). Different cross-peaks due to exchange contributions between two different sites can also be detected in the NOESY spectrum. These cross-peaks are related to the NH(10)/NH2(2) --- HOD exchange.

Information on the extent of dipolar interactions can be used as experimental "constraints" in theoretical energy minimization calculations. The presence of an exchange process selectively restricted to the NH(10)/NH2(2) --- HOD protons suggests the involvement of other exchangeable nuclei such as NH(18) in non covalent interactions (e.g. hydrogen-bonds), important for the stabilization of the conformation of folic acid in solution. Further evidence of the slow chemical exchange process of NH(18) with respect to NH(10)/NH2(2) can be obtained from saturation transfer experiments.

By irradiating the HOD resonance for a sufficient period of time, with a selective frequency, a strong reduction in signal intensity is observed on protons involved in exchange phenomena. The experimental findings show that the NH(10)/NH2(2) signal is drastically affected by HOD saturation whereas NH(18) does not show significant intensity variation. This is further evidence of the importance of NH(18) in the stabilization of selective conformation by hydrogen bond with the C(23) carboxyl group of glutamic acid. This hypothesis is confirmed by the selectivity in NH(18)-H(21) dipolar connectivities observed in the NOESY spectrum. In fact a specific NH(18)-H(21b) cross-peak is observed, suggesting the stabilization of a unique conformation in solution.

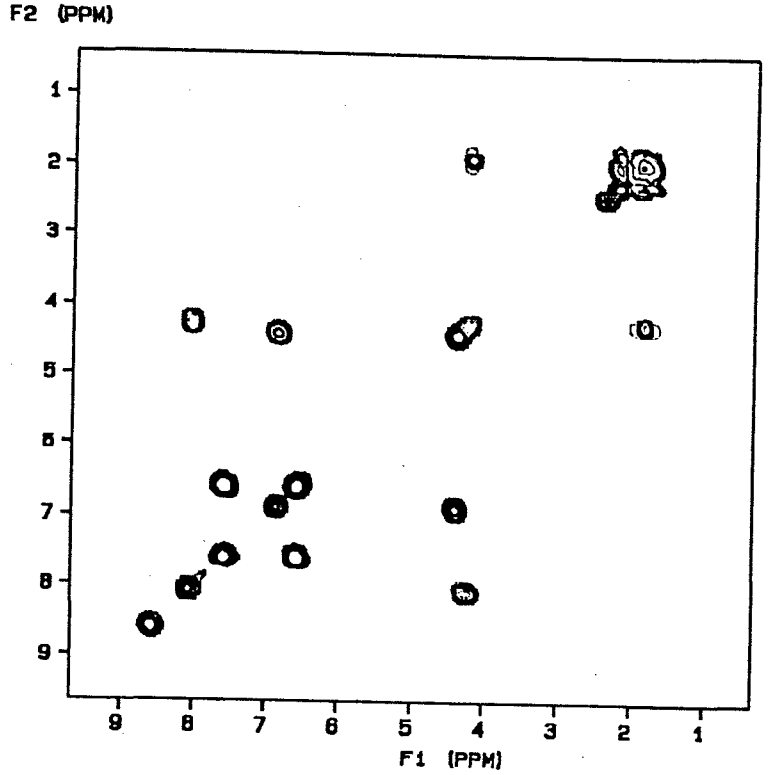

Figure 2: COSY spectrum of 0.12 mol.dm^{-3} folic acid DMSO-d$_6$ solution at 27°C.

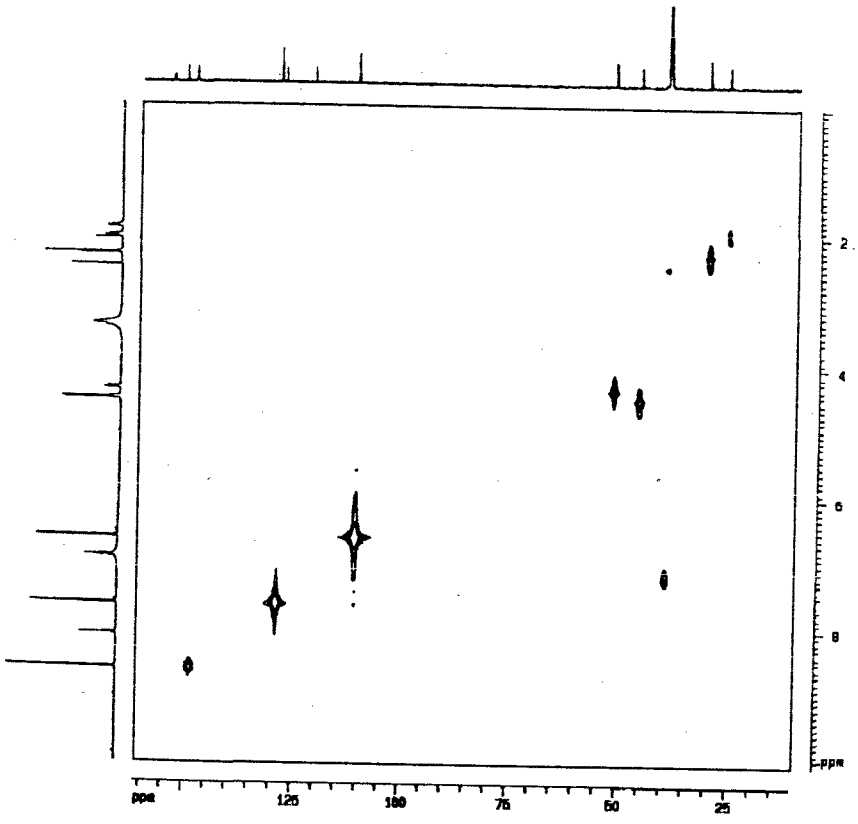

Figure 3: ^{1}H-^{13}C Hetero correlation spectrum of folic acid in DMSO-d$_6$ solution at 27°C.

Table 1

Proton and carbon NMR parameters of 0.12 mol.dm^{-3} folic acid solution at 27°C.

Nuclei	^{1}H δ ppm	^{13}C δ ppm	R_1C s^{-1}	R_1NS s^{-1}	R_1SE s^{-1}
2	--	156.160	0.11	--	--
4	--	161.274	0.21	--	--
4a	--	127.945	0.12	--	--
6	--	148.610	--	--	--
7	8.75	148.610	--	0.77	0.75
8a	--	153.823	0.55	--	--
9	4.59	45.922	11.9	5.40	5.30
10	7.02	--	--	2.80	--
11	--	150.793	0.73	--	--
12	6.74	111.216	6.12	2.00	1.46
13	7.75	128.998	5.70	1.87	1.34
14	--	121.321	0.43	--	--
15	7.75	128.998	5.70	1.87	1.34
16	6.74	111.216	6.12	2.00	1.46
17	--	166.438	0.38	--	--
18	8.22	--	--	4.71	--
19	4.44	51.762	6.07	1.62	1.17
20	--	173.744	0.37	--	--
21A	2.15	26.045	11.75	5.50	5.40
21B	2.01	26.045	11.75	5.55	5.40
22	2.42	30.439	11.60	5.00	4.90
23	--	173.932	0.30	--	--

Further evidence of the structure assumed by folic acid can be obtained from molecular mechanics calculations using the Macro Model program and experimental NMR constraints, a low energy conformation of -135.5 KJ/mol was computed after several iterative and minimizzation cycles.

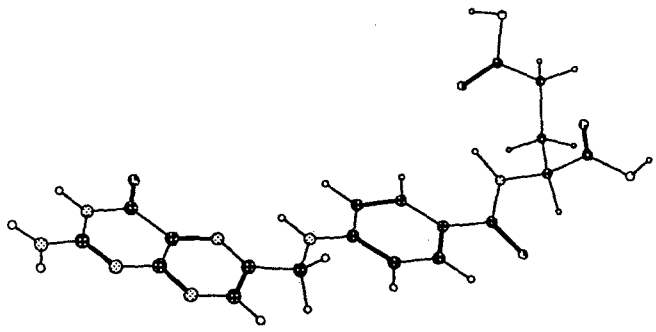

Figure 4: Solution structure of folic acid as determined by NMR experimental constraints and subsequent energy minimization calculations.

The minimized structure calculated (Figure 4), shows a NH(18)-C(19)-C(21)-H(21b) torsion angle of 315°. This is in agreement with the experimental NOESY data and confirms the conformation of folic acid stabilized in solution by the NH(18)-C(23) hydrogen bond.

4 References

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