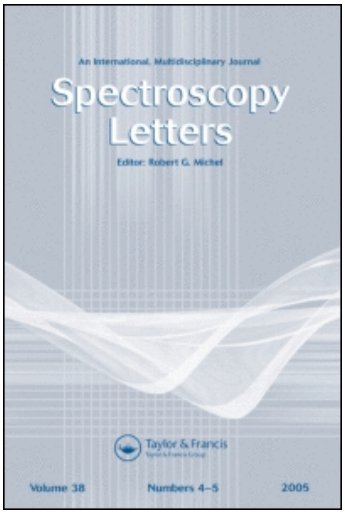

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Folic Acid: Solution Structure and NMR Strategy for Conformational Analysis

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FOLIC ACID: SOLUTION STRUCTURE AND NMR STRATEGY FOR CONFORMATIONAL ANALYSIS

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Abstract

The solution structure of folic acid was determined by the combined use of NMR experimental results, mechanics and dynamics calculations, and theoretical simulation of NMR spectra. The solution structure of folic acid showed a different molecular rearrangement to the X-Ray conformation. In particular, we identified a hydrogen bond which stabilizes a folded glutamic acid molecular moiety between the $NH_{(18)}$ and the $C_{(23)}$ carboxylic group. A different spatial conformation of the phenyl ring was also found.

In spite of its importance in the enzymatic reactions fundamental of DNA and aminoacid synthesis and its role in all redox processes, the solution structure of folic acid has not been investigated in depth. Important data on the structure of folic acid (FA) was obtained by D. Mastropaolo et al[1]. with the definition of the solid state conformation of FA crystals. X-Ray diffraction studies show that folic acid has an extended configuration stabilized by hydrogen bonds with two symmetry-related molecules and two water molecules[1].

In this paper we report data on the conformation of folic acid in solution obtained by an approach based on nuclear magnetic resonance and molecular mechanics. The dynamical properties of

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}	$\tau_c \times 10^{10}$ s
2	--	156.160	0.11	--
4	--	161.274	0.21	--
4a	--	127.945	0.12	--
6	--	148.610	--	--
7	8.75	148.610	--	--
8a	--	153.823	0.55	--
9	4.59	45.922	11.9	2.98
10	7.02	--	--	--
11	--	150.793	0.73	--
12	6.74	111.216	6.12	3.1
13	7.75	128.998	5.70	2.9
14	--	121.321	0.43	--
15	7.75	128.998	5.70	2.9
16	6.74	111.216	6.12	3.1
17	--	166.438	0.38	--
18	8.22	--	--	--
19	4.44	51.762	6.07	3.05
20	--	173.744	0.37	--
21A	2.15	26.045	11.75	2.92
21B	2.01	26.045	11.75	2.92
22	2.42	30.439	11.60	2.89
23	--	173.932	0.30	--

folic acid in solution were analyzed and the proton dipole-dipole interactions investigated in order to define the experimental proton connectivities of the molecules. Molecular mechanics calculations based on the experimental NMR findings were then used to refine the structure in order to define a model for generating theoretical 2D-NOESY spectra. Comparison of experimental and theoretical NOESY was used as a criterion to assess the validity of the proposed solution structure.

The complete proton and carbon assignments obtained in DMSO-d_6 are reported in Table 1. They are in good agreement with the results obtained in D_2O at pH=13[2-3]. Conventional COSY and Hetcor experiments were used to assign the proton and protonated carbons

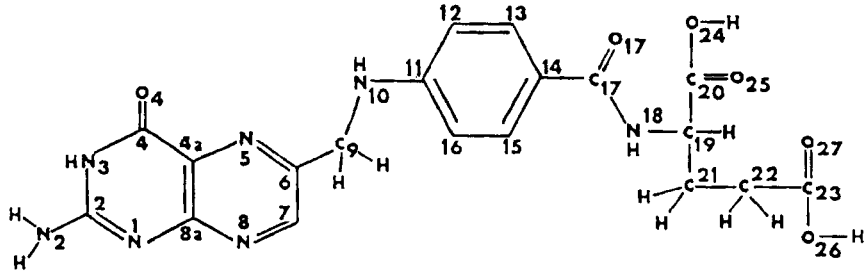

Figure 1 - Structure and numbering of folic acid.

respectively. Selective proton-carbon nuclear Overhauser effects were used to assign the quaternary carbons[4-6]. Carbon spin-lattice relaxation rates were used to calculate the effective correlation time, τ_C, modulating the proton-carbon interactions. The experimental carbon spin-lattice relaxation rates and the correlation time τ_C, calculated on the basis of Allerhand's[7] equation are also reported in Table 1.

In order to investigate macromolecule solution structures, the strategy shown in Figure 2, based on experimental NMR parameters, molecular mechanics and dynamics calculations and NMR simulations was used. The first step requires characterization of the fundamental NMR spectral parameters, the most important of which are the proton and carbon chemical shifts. The definition of chemical shift assignments enabled investigation of the dynamical properties of solution structure, by spin-lattice relaxation rate analysis of protonated carbons[8]. The dynamical information can be also obtained from selective, non-selective and multiselective proton spin-lattice relaxation rates[9]. Careful investigation of molecular dynamical behaviour gave some preliminary structural indications; infact, isotropic molecular motion is very often associated with specific "structured" conformations stabilized by non-covalent interactions. Anisotropic motion and jump rotations of groups and residues are related to higher dynamical degrees of freedom and are typical of random structures.

The rotational correlation times (Table 1) calculated from protonated carbon spin-lattice relaxation rates indicate that folic acid is characterized by isotropic motion and suggest that it has a specific structure in solution.

More detailed information about the conformational properties of a molecule in solution can be derived from combined analysis of NMR experiments involving internuclear dipolar connectivities: geometrical constraints can be obtained if selective parameters can be measured. This is possible in one- and two-dimensional experiments. In one-dimensional, experiments selective perturbation pulse sequences are used to induce selective magnetization components from which specific internuclear distances can be calculated[4,9-11]. In two-dimensional experiments, i.e. NOESY and ROESY, the complete network of dipolar internuclear interactions can be observed[12,13]. Provided that detailed dynamical analysis is performed, one-dimensional selective experiments allow the direct determination of internuclear distances[14]. Similarly, two-dimensional experiments, NOESY and ROESY, used under different conditions of molecular motion can provide a description of molecular geometry and the derivation of specific constraints between nuclei within a radius of 5Å[15].

The derivation of the experimental internuclear distances and "constraints" on folic acid was based on selective proton-carbon nuclear Overhauser effects, {H}C-NOEs, and NOESY spectra respectively. The most interesting features detectable from NOESY spectra were: i) the presence of connectivities between nuclei only a few bonds distance which confirm previous assignments; ii) the presence of dipolar interactions between closely related nuclei of primary importance for the identification of specific structures; iii) the presence of cross-peaks between exchangeable nuclei.

The most important results obtained with FA, are the identification of a cross-peak between the $NH_{(18)}$-$H_{(21b)}$ protons, and the absence of an exchange cross-peak for the $NH_{(18)}$. This suggests that the $NH_{(18)}$ proton is involved in a hydrogen bond with the carboxylic group of glutamic acid.

As shown in Figure 2, the generation of molecular models as an intermediate step for theoretical NMR calculations requires a massive set of structural data. In order to simplify this procedure, molecular mechanics and dynamics calculations should use a known model as starting structure, e.g. the solid state conformation.

From the X-Ray structure of folic acid, an entire set of conformers was generated, exploring conformational space by "Internal Coordinate Monte Carlo Method"[16], and then the lower energy structure was fully energy minimized by the MM2 force field[16-19]. In Figure 3 are shown the structures obtained by sampling selected conformations during a molecular dynamics simulation[19]. From Figure 3 the region of folic acid in which the loop structure is maintained by hydrogen bond is shown.

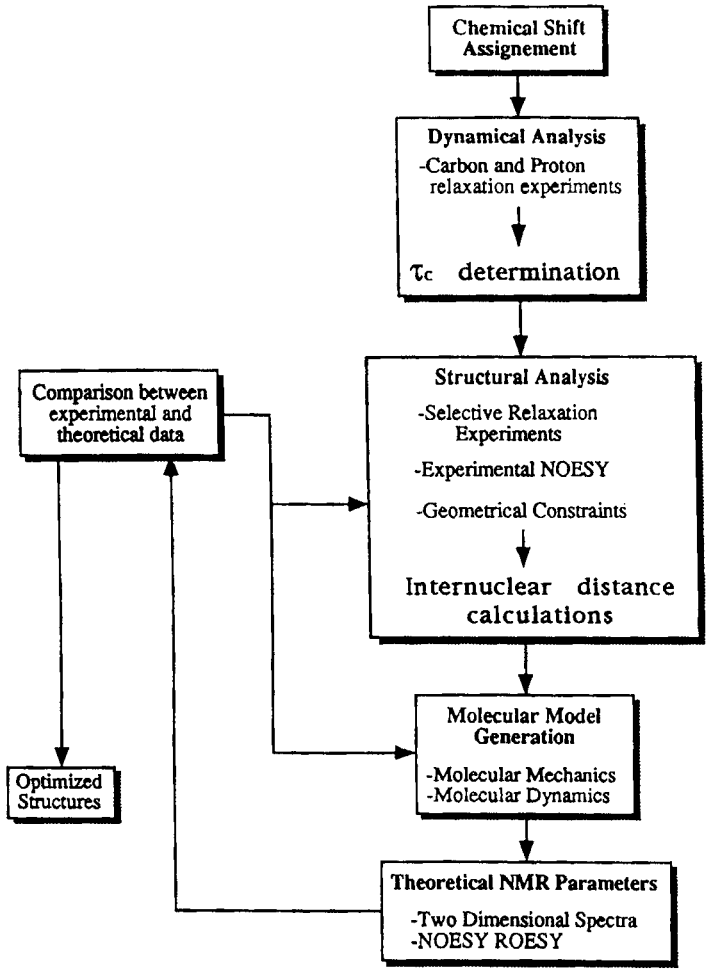

Figure 2 - Proposed strategy for solution structure investigation based on experimental NMR results and theoretical calculations.

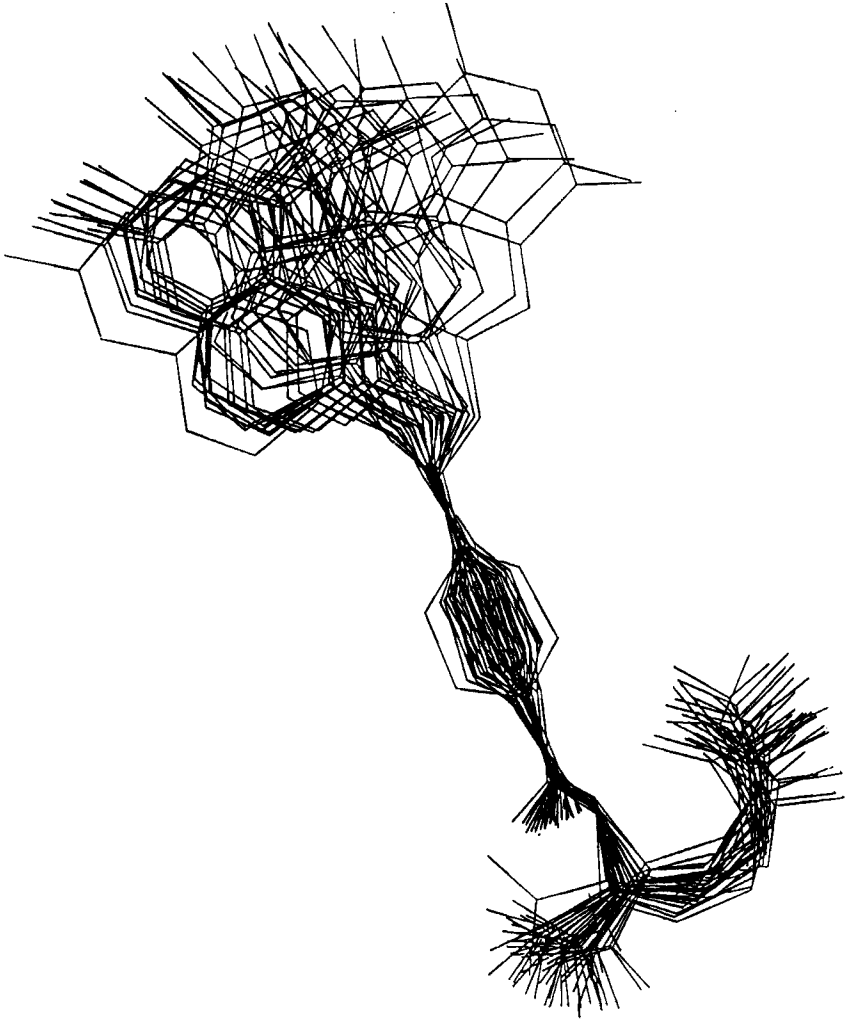

Figure 3 - Family of energy minimized structures computed by molecular dynamics calculations.

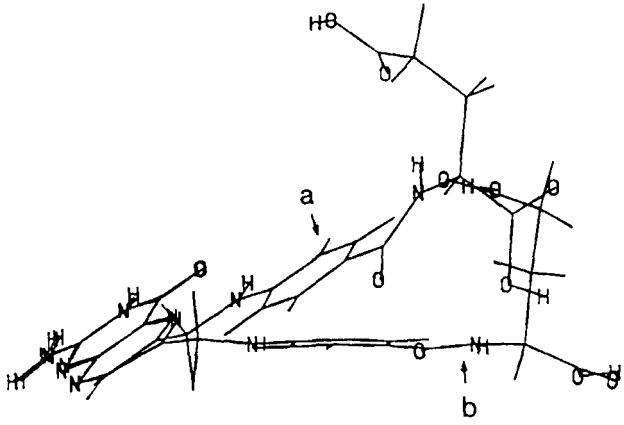

Figure 4 - Folic acid solution structure with superimposed crystal structure determined by X-Ray diffraction. a) X-ray structure, b) energy minimized structure.

The most stable conformation was characterized by a low energy value of -135.5 KJ/mol and showed a loop structure stabilized by the $NH_{(18)}$-$C_{(23)}$ carboxyl hydrogen bond. A torsion angle value of 315° was observed between the $N_{(18)}$-$C_{(19)}$-$C_{(21)}$-$H_{(21b)}$ nuclei. Intermolecular distances of 2.69Å and 3.77Å between $NH_{(18)}$-$H_{(21b)}$ and $NH_{(18)}$-$H_{(21a)}$ respectively (in agreement with experimentally detected NOESY cross-peaks) were verified in the energy minimized structure.

The most evident differences between the structure in solution determined by combining theoretical and experimental results[20] with the crystal structure are shown in Figure 4. The two structures differ mainly in the arrangement of the glutamic acid moiety. A different spatial conformation of the phenyl aromatic ring is also shown.

The family of structures obtained by molecular dynamics could be used to generate theoretical two-dimensional NOESY and ROESY spectra. In the present study the lower energy conformational model was used to calculate the NOESY spectrum with the CORMA (COmplete Relaxation Matrix Approach) program[15,21] .

This algorithm considers the evolution of the magnetization during the mixing period of the NOESY experiment described as[22]:

$$\partial \mathbf{M} / \partial t = -\mathbf{R}\,\mathbf{M} \qquad [1]$$

where **M** is the magnetization vector decribing the deviation for thermal equilibrium and **R** is the matrix describing the complete dipole-dipole relaxation network. In the **R** matrix the diagonal elements are the longitudinal and the off-diagonal elements the cross, relaxation rates. Equation [1] has the solution:

$$\mathbf{M}(\tau_m) = \mathbf{a}(\tau_m)\mathbf{M}(0) = e^{-\mathbf{R}\tau_m}\mathbf{M}(0) \qquad [2]$$

where **a** is the matrix of "so-called" mixing coefficients which are proportional to the measured NOESY intensities.
Comparison of CORMA and experimental NOESY spectra provided a very accurate indication of the distance between the calculated conformation and the structure in solution. As shown in Figure 2, an iterative procedure can be used to increase the convergence between the CORMA and experimental spectra[23]. This procedure involves modification of the theoretical model, however in our opinion the sole use of a minimized structure cannot usually produce a very accurate fit between experimental and calculated data. A more realistic description of the structural behaviour of a biomolecule in solution can be obtained if the complete set of information derived from molecular dynamics is considered.
This approach and new algorithms including simulation of NOESY and ROESY spectra from energy minimized structures and from a family of conformations obtained by molecular dynamics calculations, can further increase the descriptive reliability of biomolecule behaviour in solution.

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