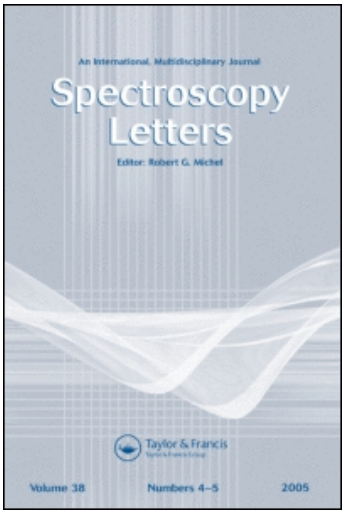

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A ^{1}H Paramagnetic Relaxation Study on the Interaction of Peptides with Aminoxyl Spin Labels in Apolar Environments

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A ^1H PARAMAGNETIC RELAXATION STUDY ON THE INTERACTION OF PEPTIDES WITH AMINOXYL SPIN LABELS IN APOLAR ENVIRONMENTS

Key words: NMR, proton relaxation, paramagnetic probes

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INTRODUCTION

The use of stable free radicals such as aminoxyl spin-labels has been recently exploited for obtaining information on solution structures of peptides [1,2] and proteins [3,4] from multidimensional NMR spectra. In those reports, it has been suggested that an efficient spin-labelling of water and dimethylsulphoxide (DMSO) solvents may be achieved and the paramagnetic solvent yields relaxation effects on backbone proton nuclei which are directly driven by the molecular surface accessibility. Upon the addition of the paramagnetic probe, the observation of absence in chemical shifts changes and of nuclear relaxation effects which are fully rationalised in terms of the solution structure, support a dynamic model for the interaction between the chemical probe and the biomolecule where weak collisional adducts favour the dipolar coupling of the unpaired electron with the outer nuclei of the investigated molecule.

Most natural cyclopeptides are of great interest because of their biological properties. Cyclization of the backbone chain

reduces the number of allowed conformations leading to rigid molecules often more specific and efficient as well as resistant in vivo versus host exopeptidases.

Cyclo L-Ala-D-Ala-L-Aeo-D-Pro (HC toxin), a host-specific phytopathogen metabolite of Helminthosporuim carbonum with a well defined and rigid structure in chloroform solution[5] has been therefore chosen as a model system to investigate the possibility of using the spin labelling approach in low polarity solvents for a structural characterization of biopolymers active in non aqueous environments and for obtaining additional information on the dynamics of the interaction between the paramagnetic probe TEMPOL with peptides and proteins.

MATERIALS AND METHODS

HC toxin was synthesized as reported in ref. 6. All the NMR measurements were achieved on a Bruker 200 AC spectrometer, equipped with an Aspect 3000 computer.

A 1D reference spectrum was obtained from a 15 mM solution of HC toxin dissolved in $CDCl_3$ (Sigma) containing TMS as internal standard for chemical shifts. To the initial sample progressively increasing amounts of 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1N-oxyl (TEMPOL, Sigma) were added to yield final nitroxide concentrations 20 and 30 mM respectively.

A total of 64 scans were collected per sample, using a sweep width of 3500 Hz and a data size of 16 K words.

2D COSY spectra consisting of 512 increments of 1024 data points were acquired in the absence and in presence of TEMPOL 30 mM. Sine and cosine-bell functions were applied respectively in t2 and t1 dimension; the experimental data were then zero filled to 2K in t1 before Fourier transform.

All measurements were performed in the same experimental conditions and the data were all processed with the same parameters to allow for quantitative comparison of peak intensities.

Modelling and restrained molecular dynamic simulation were carried out with Sybyl software (Tripos Ass.) running on a SUN Spark 4/330 workstation. Graphic interface for IBM PC was supplied by NITRO software (Tripos Ass.).

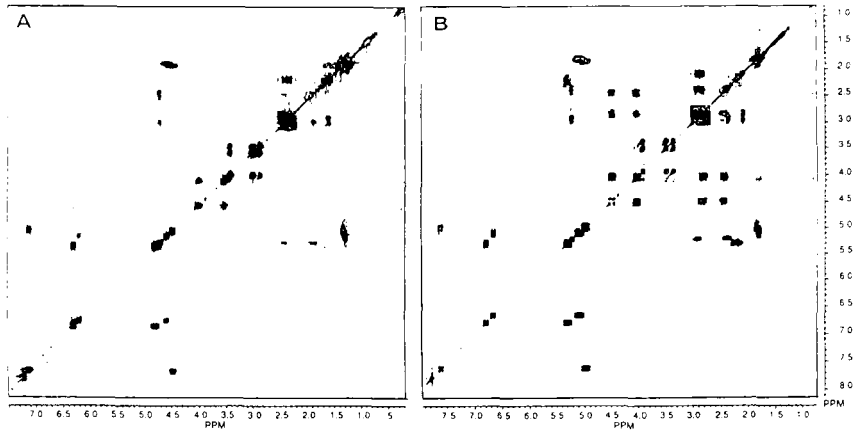

Fig 1 - Magnitude 2D COSY spectra of HC toxin : (A) in the presence of 30 mM of TEMPOL; (B) diamagnetic solution

A preliminary molecular model was built on the basis of the ϕ and ψ torsional angles derived from vicinal Hα-NH J couplings reported by Mascagni et al. [7]

After 100 cycles of energy minimization using the Kollman force field a total of 50 psec of restrained molecular dynamic were performed. A Boltzmann distribution of starting velocities was imposed at 50°K. After 10 psec of thermal equilibration at 300°K the 'ramping temperature' methodology was employed, alternating 5 steps of 10 psec at 300° and 500° K.

RESULTS AND DISCUSSION

Helminthosporium carbonum race 1, the causal agent of a loaf spot disease on maize, produces the cyclic tetrapeptide L-Ala-D-Ala-L-Aeo-D-Pro, better known as HC toxin, responsible of the pathogen effects on susceptible protoplasts [8] . The molecule contains two D-amino acids (D-Ala2 and D-Pro4) and an unusual residue , Aeo (2-amino-9, 10-epoxy-8-oxodecanoic acid), necessary for the toxicity[9].

Structural characterization by X-ray crystallography and NMR of HC and related toxins has been reported [7,10,11]. There

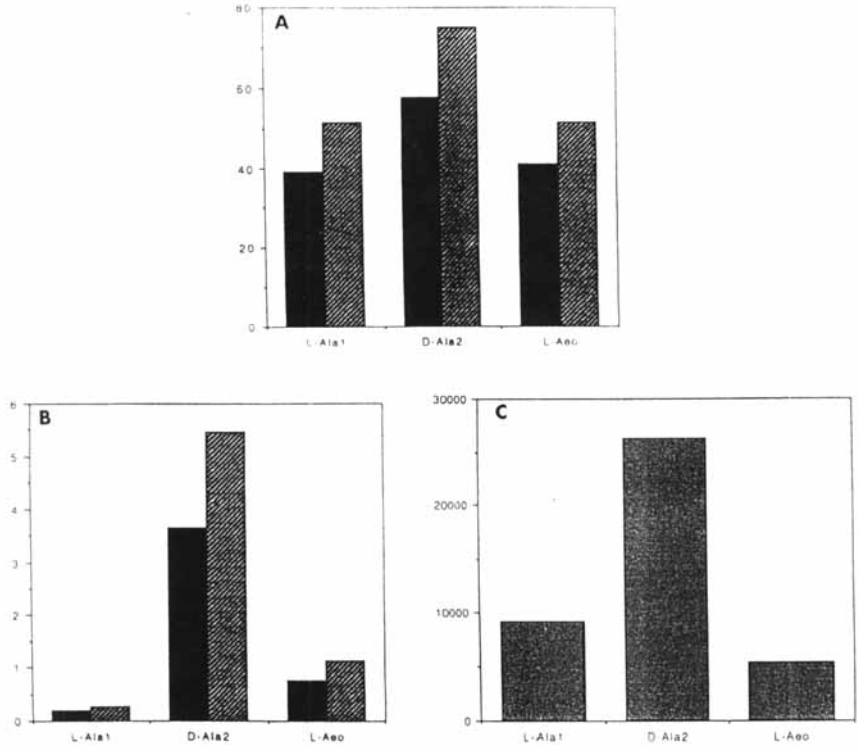

Fig. 2 - A) Effects on chemical shift upon Tempol addition : ■ = Tempol 20 mM ; ▣ = Tempol 30 mM. B) Corresponding effects upon DMSO addition: ■ = DMSO 20 mM ; ▣ = DMSO 30 mM (from ref 13 , modified); C) Paramagnetic attenuation of NH - Hα COSY cross-peak volumes in the presence of Tempol 30 mM, expressed as Vd/Vp (Vd = diamagnetic volume; Vp = paramagnetic volume)

is a general consensus that these molecules adopt in solution a bis γ turn conformation stabilized by two intramolecular hydrogen bonds . More recently for HC toxin a slightly different structure in chloroform has been proposed (12) in which the presence of a third γ turn leads to an additional hydrogen bond between D-Pro4 CO and D-Ala2 NH.

All the NMR data indicate also a differential exposure of the amide protons: a general shielding is observable for Ala1 and Aeo NH, while Ala2-NH shows higher accessibility to solvent molecules .(5)

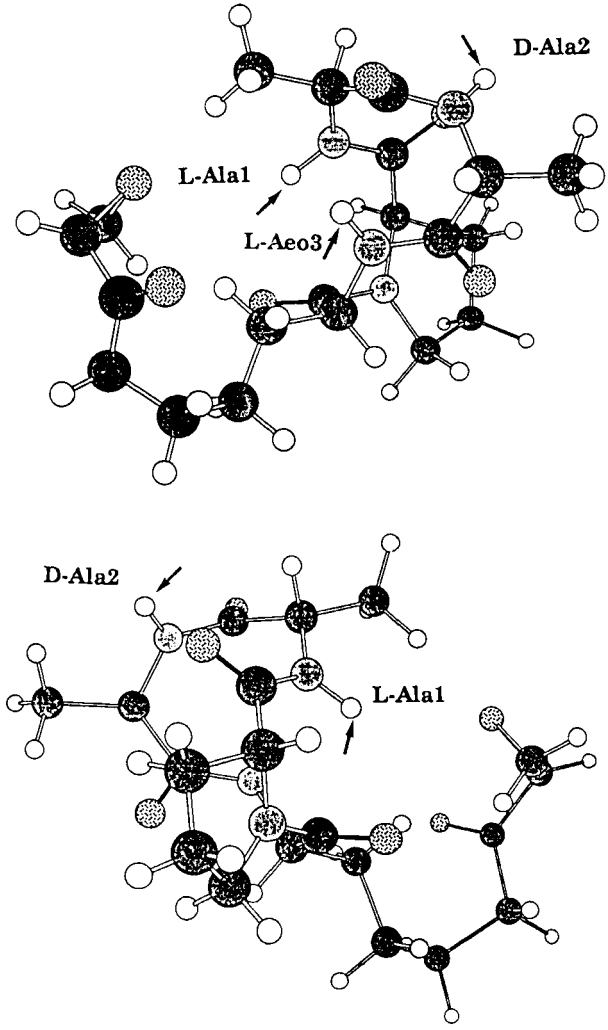

Fig. 3 - Strucure proposed for HC toxin in chloroform. The molecule is shaded by atom type:

○ = Hydrogen; ● = Carbon; ◌ = Nitrogen; ⊗ = Oxygen

In Fig. 1 the COSY spectra obtained for HC toxin in the presence and in the absence of TEMPOL are shown. Resonance assignment was made according to Pope et al .[13]

From a direct comparison of the two spectra, it is apparent that the spectrum recorded for the paramagnetic solution has a reduced number of J connectivities, in agreement with previous studies .[2-5] Furthermore, the NH-Hα cross-peaks have a differential intensity decrease. From the quantitative analysis of the paramagnetic attenuations, shown in Fig. 2, the high TEMPOL exposure of Ala2 backbone proton correlations is easily seen. This feature is consistent with the backbone hydrogens surface accessibility found in the molecular model reached after restrained dynamic simulation and shown in Fig. 3.

This three-dimensional structure was obtained using as constraints a set of upper and lower distance limits ($r \pm 0.5$ Å) centred around the values calculated from homo and heteronuclear NOE measurements on Val3-HC toxin .[14]

Amide proton chemical shift changes, proportional to the TEMPOL concentration, not observed in other similar structural investigations, is also apparent, see Fig. 4. The possible explanation for this finding of an amynoxyl induced conformational change can be ruled out by the substantially constant vales of the $^3J_\phi$'s during the spin-label titration (maximum variation of 0.6 Hz).

A comparison of the reported chemical shift changes upon DMSO additions [7], suggest that the enhanced hydrogen bond acceptor capability of the paramagnetic probe in respect to the solvent molecules can account for the observed effects. Moreover, as the shift effects caused by DMSO are approximately one order of magnitude lower than those measured for the aminoxyl, a general assessment can be made about the dynamics of the TEMPOL-peptide interaction: the hydrogen bonding of the paramagnetic molecule *via* the N oxyl moiety may play an important role in the intermolecular interaction process.

The bifunctional nature of TEMPOL as hydrogen bond acceptor due to concomitant presence of the alcoholic function

could explain the increased effects on chemical shift in respect to DMSO.

However, the extent of the generalized interaction is not large enough to efficiently compete with polar solvent molecules, as in the previously referred investigations[1,2,4], carried out in water and DMSO solutions, where a sizeable chemical shift change has never been detected.

According to the molecular structure shown in Fig. 3, the residual intensities of NH-Hα cross-peaks reflect the surface accessibility of the relative hydrogens.

The good correlations observed between paramagnetic attenuation data and exposure degree suggest that the spin-labelling of solvent of low polarity with chemical probes such as TEMPOL may yield significant information on molecular solution structures. The chemical shift changes, induced by the additions of the paramagnetic probe, are conformationally driven and proportional with those obtained for DMSO.

Thus, a general use of the TEMPOL paramagnetic probes for determining the backbone hydrogen surface accessibility can be proposed independently on the polarity of the solvent where the structural investigation is carried out.

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