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[*] In Kazan University the Electron Paramagnetic Resonance (EPR) was discovered by Zavoisky E.K. in 1944.

NMR studies and molecular dynamics simulation of cyclosporin in complex with detergent micelles[†]

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Cyclosporin A is a highly hydrophobic peptide, but its complex with sodium dodecyl sulphate micelles can be readily dissolved in water. Nuclear magnetic resonance (NMR) investigations of cyclosporin bound to detergent micelles were carried out (including NOE spectroscopy) and yielded internuclear distances for a set of atom pairs. Based on these structural data, conformation of cyclosporin was obtained by means of molecular dynamics simulation.

1. Introduction

Cyclosporin A (CsA), cyclo(-Bmt-Abu-Sar-Mle-Val-Mle-Ala-DAla-Mle-Mle-Mva-), is a peptide used in therapeutics as an immunosuppressant. It acts through formation of a complex with the 18-kDa protein cyclophilin (Cyp18). This protein is ubiquitous, and the CsA–Cyp18 complex accumulates in cytoplasm in many types of cells; however, cyclosporin influences selectively T-cells. Apparently, cell recognition occurs, involving receptor proteins on cell membranes [1].

The task of determination of the structure of a molecule bound to a cell membrane is thus of a great importance. Phospholipid vesicles are the best structures representing cell membranes *in vitro*; however, NMR-based structure determination for molecules bound to heavy vesicles is hampered due to short transverse relaxation times. Fortunately, useful information can be obtained if relatively small supramolecular structures, detergent micelles, are employed [2, 3].

Stable conformations of cyclosporin, which exist in apolar solvents, were studied by means of NMR and different computational approaches [4, 5]. More recently, the question of interaction of CsA and metal ions within detergent micelles was studied [6]. Structure of this peptide is found to be flexible and strongly dependent on the environment (solvent polarity, presence of ions, complex formation with larger molecules [7-9]). This fact calls forth wide experimental study of CsA.

2. Experimental

Cyclosporin A ($\geq 98.5\%$) was purchased from Sigma-Aldrich, Inc. and used without further purification. Sodium dodecyl sulphate in concentration exceeding the critical micelle concentration was used as the model-mimicking compound. Spectra were recorded on a Bruker Avance II spectrometer operating at the proton frequency of 500 MHz; heteronuclear measurements and some of the NOESY experiments were performed on a Bruker Avance at 700 MHz (Leipzig University). For the sake of signal assignment, heteronuclear correlation spectra were acquired: HSQC, HMBC, and band-selective HMBC. The latter excited carbonyl resonances in a relatively narrow band (3522 Hz, corresponding to 20 ppm in the ^{13}C dimension). Geometric restraints, extracted from the NMR data,

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were then employed as input data in molecular dynamics (MD) simulation. Ensembles of structures were built in the XPLOR-NIH 2.29 programme [10]. One hundred structures were generated in the simulated annealing protocol (6000 iterations at the initial temperature of 800 K, then cooling during 14000 iterations down to 100 K).

3. Results and Discussion

One-dimensional ^{1}H NMR spectrum of cyclosporin is shown in figure 1. Evidently, four resonances are dominant in the amide region (6.5-8.5 ppm); seven lines of NCH_3-groups are notable in the region 2.7-3.6 ppm. Signals of amide protons are absent in the spectrum recorded in D_2O due to intermolecular exchange with deuterium; signals of α-protons are suppressed in the H_2O/D_2O sample together with the solvent resonance. One conformation is thus dominant, and we can assign the signals using two-dimensional correlation experiments TOCSY, ($^{13}C,^1H$)-HSQC, ($^{13}C,^1H$)-HMBC, and NOESY.
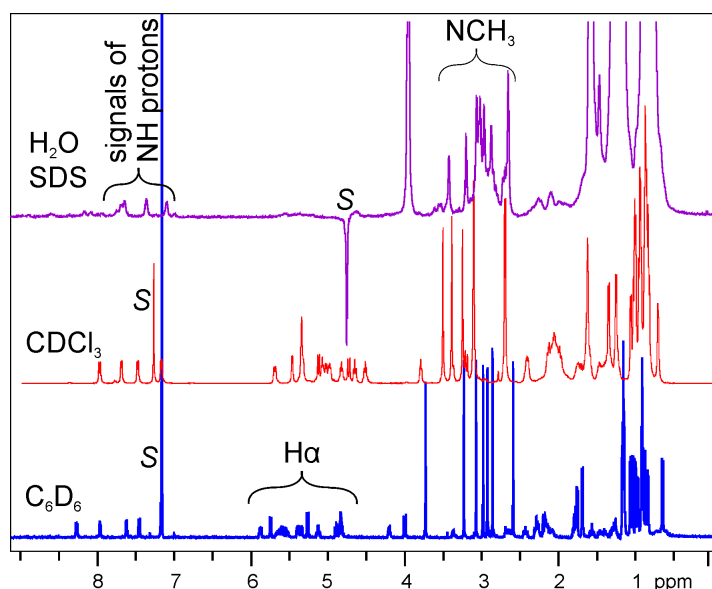

Figure 1. ^{1}H NMR spectra of cyclosporin at 20°C. From top: the CsA–SDS micelles system, spectrum with water suppression; micellar solution based on D_2O; chloroform. Solvent signals are marked by "*S*".

Increase in temperature causes small changes in the amide resonances. The signal of Abu[2] shifts significantly to the higher field (0.018 ppm/K in the range from 10 to 35°C), what is interpreted usually as an evidence of outward NH bond, preventing the formation of an intramolecular hydrogen bond [11]. Alanine NH signal has the temperature gradient of 0.005; the other two protons (D-Ala[8] and Val[5]) show $\Delta\delta/\Delta T < 0.003$ ppm/K, which may be an evidence of intramolecular hydrogen bonds involving the latter two protons. The minor signals also move to the higher field in a similar manner. Their intensity remains the same.

Some information on the spatial structure of a molecule is contained in chemical shifts. Systematic deviations in signals of backbone protons to the higher or lower field mean that corresponding backbone region is a part of an α- or β-structure, respectively [12]. On a qualitative level, comparison of ^{1}H and ^{13}C spectra can give an evidence of similarity or dissimilarity of molecular conformations in different solvents. Proton spectra are sensitive to local environments (including solvent effects), while ^{13}C resonances depend mainly on local conformations. In general, ^{13}C NMR spectra of CsA in apolar solvents and in micellar solution are similar (fig. 2). The sequence of the C^α signals in all three media is this: residue 1 (formally also 12) is the leftmost, then we observe 11 and 10; closely located three signals 4, 5, and 6; after an empty interval of ~5 ppm stand signals of residues 3, 2, 7, 9, and 8. Proton spectra, despite their narrow frequency range and relatively big variations in chemical shifts, can also be identified. For instance, the α-proton of Mle[9] resonates always in the lowest field, while that of Ala[7], in the highest field. We can thus expect that local conformations of each amino acid residue undergo subtle changes when going from one apolar medium to another. The same kind of information is provided by solid-state NMR spectra. In particular, similarity of ^{13}C NMR spectra recorded in solid-

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state and in solution proves existence of a single stable conformation in the liquid state [13]. In our case, comparison of the spectra seems difficult due to a large number of lines; nevertheless, arrangements of the peaks in organic solvents and in crystal are similar (fig. 3).
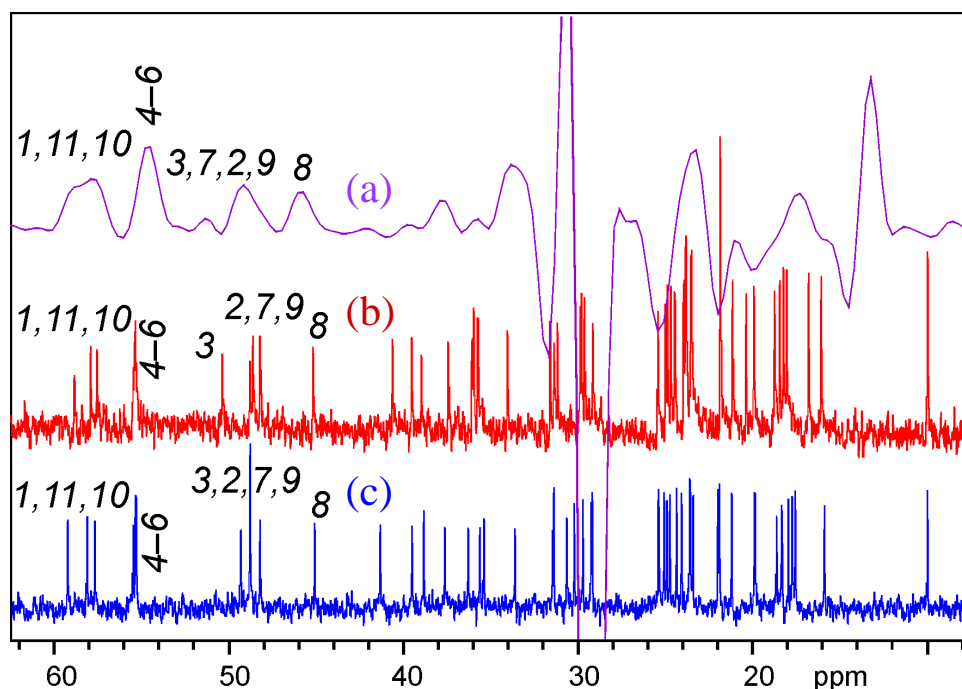

Figure 2. Comparison of ^{13}C spectra of CsA in three media: (a) complex with SDS micelles (projection from an HSQC spectrum); (b) chloroform; (c) benzene.
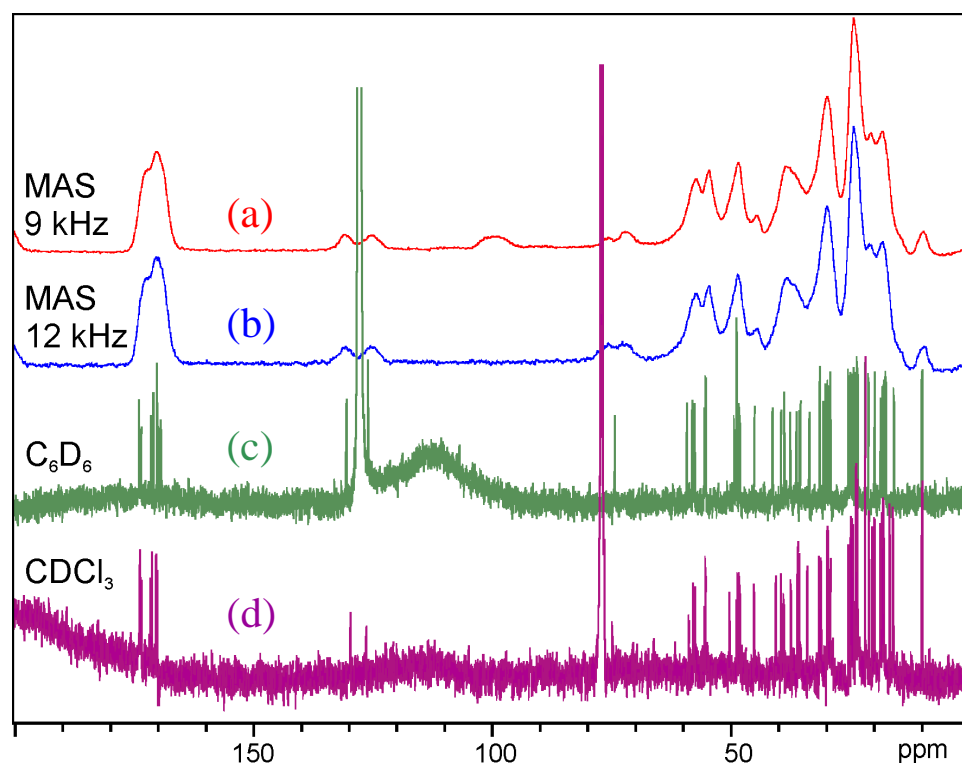

Figure 3. Comparison of ^{13}C spectra of CsA in three media: (a and b) CP-MAS recorded with different rotation speeds; $^{13}C\{^1H\}$ spectra in benzene (c) and chloroform (d).

Two-dimensional NOE spectroscopy provides information for constructing a model structure of cyclosporin. Analysis of NOESY spectra was made following [14-16]. Intensity of a cross-peak of the nuclei a and b is normalized by intensities of corresponding diagonal peaks (standing in the cross-section with the same indirect frequency F1). After that, dependencies of intensities on the mixing time $I(F_{1a}, F_{2b}) / I(F_{1a}, F_{1a}) = f(\tau_m)$ are built. Atom pair $Ala^7(H^\alpha\text{-}H_3^\beta)$ was chosen for calibration. Corresponding distance is 2.8 Å, according to the jump model of averaging [17]; simpler averaging models yield lesser values (by 0.1-0.2 Å). For the NCH_3-groups, averaging $R = \langle R_{ij}^{-3} \rangle^{-1/3}$ was used (possible variants are described in [18]).

One hundred structures were generated in the simulated annealing protocol; ten of them, having minimal energies, were selected. They are moderately close to each other; RMSDs between the lowest-energy structure and remaining nine molecules in positions of backbone atoms lie from 3.1 to 4.8 Å. Some violations were observed: $Mle^{10}(H^\alpha)$-$Mva(H^\beta)$, $Mle^{10}(NCH_3)$-$Mle^9(H^\alpha)$ (after averaging the distances to the methyl protons), $Mle^6(NCH_3)$-$Ala(H^\alpha)$, and $Sar(NCH_3)$-$Bmt(H^\alpha)$ were all longer that the NOE-based values. Analysis of NOEs underestimates the distances due to spin diffusion, when the coherence is transferred between atoms a and b via one or several intermediate atoms; the apparent cross-relaxation rate has no clear correlation with the distance r_{ab} in this case. We repeated the simulation, having omitted the incorrect distances. The new ten structures with minimal energies showed backbone RMSDs from 2.6 to 4.6 Å. Figure 4 presents one of them (with the lowest energy) compared with the structure of cyclosporin in chloroform, modelled allowing for hydrogen bonds. This simulation was performed as described in [19]; information on hydrogen bonds was taken from literature data [7].
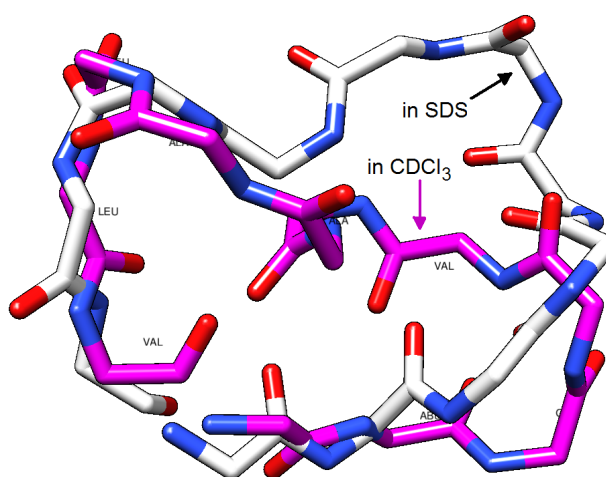

Figure 4. Comparison of the CsA structures based on NOE data obtained in chloroform and in complex with SDS micelles. Illustration was prepared in the Chimera programme [20].

4. Conclusions

Several conformers of cyclosporin coexist in micellar solution based in SDS. Structure of the main conformer was determined with the aid of the NOESY method. The peptide ring is nearly planar; some differences compared to its structure in apolar organic solvents were found. Close contacts of Bmt H^α with α-protons of Mle^6 and Ala^7 are absent. The ring of cyclosporin becomes less oblate; chain regions with residues 9-11, 1 and 4-6, which in chloroform are close to each other and resemble β-structure, move off from each other in the complex. This change may be due to steric interaction with SDS molecules which prevents formation of intramolecular hydrogen bonds. Note also that H^α chemical shifts in the micellar solution are, in general, smaller that in organic solvents. It gives an evidence of additional shielding of the peptide by surrounding detergent molecules.

Acknowledgements

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