JrnlID 12668_ArtID 641_Proof# 1 - 04/05/2019

BioNanoScience
https://doi.org/10.1007/s12668-019-00641-z

Conformational Variability of Cyclosporin C Dissolved in Dimethylformamide

S.V. Efimov[1] · Yu.O. Zgadzay[1] · S. Darwish[1] · V.V. Klochkov[1]

Abstract

Action of biologically active compounds requires proper spatial structure, and sometimes controversial requirements should be fulfilled at the same time. Cyclosporin, widely used to prevent allograft rejection, is an example because it should cross through a cell membrane and form a complex with a target protein inside the cell. This difficulty is overcomed by flexibility of the molecule. Cyclosporin A was widely studied, and it is known to have multiple conformations in polar media such as water and methanol. However, detailed characterization of all these conformers is difficult: their lifetime is too long for MD simulations, analysis of NMR spectra is hampered due to severe signal overlap, and IR spectroscopy gives parameters averaged over all conformers. This paper presents characterization of conformational equilibrium of cyclosporin C dissolved in dimethylformamide. High-resolution NMR spectra recorded at 700 MHz allowed distinguishing most of observed amide proton signals. Existence of several intramolecular hydrogen bonds over the whole set of conformers was supposed; in most cases, however, these bonds are disrupted. Kinetics of a conformational transition is evaluated. Obtained results are in agreement with what is known about cyclosporin A, but can give new information on the role of additional H-bond donors (there are six of them in CsC vs. five in CsA) in the observed chain flexibility.

Keywords Dynamic NMR · Cyclosporin C · Chemical exchange · Hydrogen bond

1 Introduction

Cyclosporins, and more generally, cyclic peptides remain interesting and promising objects due to their diverse properties. Binding the tail of a peptide chain to its head leads to dramatic changes in the molecular dynamics and biochemical properties: it becomes resistive to proteolytic enzymes and to denaturation caused by high temperatures; the conformational space covered by the molecules is reduced significantly so that the affinity to target proteins increases [1]. Cyclic peptides such as gramicidin [2] or artificial compounds studied in [3] can possess amphipathic properties providing them ability to permeate into cell membranes and serve thus as antimicrobial agents.

The question of membrane permeability is of special interest since it defines the bioavailability of drugs which should find their target molecules in the cytoplasm. A recent study of cyclosporin A (CsA) using MD simulation has shown that it is conformational flexibility which allows the peptide molecule to move across hydrophobic interior of the membrane and then exit from it into aqueous environment [4].

Cyclosporin A has many natural analogues, most of which differ from CsA in one or two sites by alteration of amino acid or N-methylation state. Substitution of threonine for the aminobutyric acid (Abu2) residue yields cyclosporin C (CsC), one of congeners which still has some immunosuppressive activity, though weaker than that of CsA. It is capable of binding to cyclophilin and was shown also to have antiviral effect against vaccinia virus [5]. Whereas CsA molecule contains five potential hydrogen bond acceptors (four NH groups and one OH group in residue Bmt1), CsC has an additional hydroxyl group in Thr2. One can expect that appearing of new OH groups may rearrange the pattern of H-bonds defining the structure of the peptide ring, influencing its dynamics and hydrophobic properties. Our aim was to observe its conformational behavior in dimethylformamide as an example of a polar medium by NMR spectroscopy.

✉ S.V. Efimov
Sergej.Efimov@kpfu.ru

[1] Institute of Physics, Kazan Federal University, Kazan 420008, Russia

2 Results and Discussion

Measurements were carried out on a Bruker Avance II 500 and Avance III HD 700 spectrometers. Sample concentration was 1 mM. A series of one-dimensional ^{1}H NMR spectra was recorded at different temperatures from 14 to 40 °C. At least 20 signals of amide protons can be observed in the low-temperature spectrum obtained at the frequency of 500 MHz. Exact counting is hampered by the fact that as the temperature rises, new weak peaks appear or overlap with their neighbors, while other broadens. A short series of spectra obtained at 700 MHz allowed better observing some weak signals overlapping with the major ones due to a better spectral resolution. Finally, 31 signals were analyzed. To determine which amino acids gave these peaks, 2D TOCSY and nuclear Overhauser effect spectra were also obtained (500 and 700 MHz TOCSY at 25 °C, 500 MHz NOESY at 37 °C , and 700 MHz ROESY at 15, 25, and 32 °C). Part of the TOCSY spectrum is shown in Fig. 1.

As a result, the peaks were distinguished into nine groups containing from 1 to 5 exchange-correlated members each. Assignment, where possible, was achieved using TOCSY (correlation with two peaks in the middle of the spectrum points to Hα and Hβ of threonine; valine is recognized by its Hβ signal nearly at 2 ppm and two close Hγ peaks; alanine has a typical strong signal of β methyl group—see an example in Fig. 1). Dependence of the chemical shifts on the temperature allowed calculating the value of $\Delta\delta/\Delta T$. Results are presented in Fig. 2.

It can be seen from the spectra that many peaks belonging to the same exchanging position appear in quite distant spectral regions, which may differ by 1 and even 2 ppm; this is especially prominent in groups *a* and *b*. Second, intensities of most signals vary in a relatively narrow range, and there are a few peaks having a decreased intensity. In particular, signal no. 3 in the spectrum obtained at 25 °C (bottom spectrum in Fig. 2) has the relative intensity of 53% if the peak is 30 (in fact, overlap of several signals) is assumed to be 100%, but all

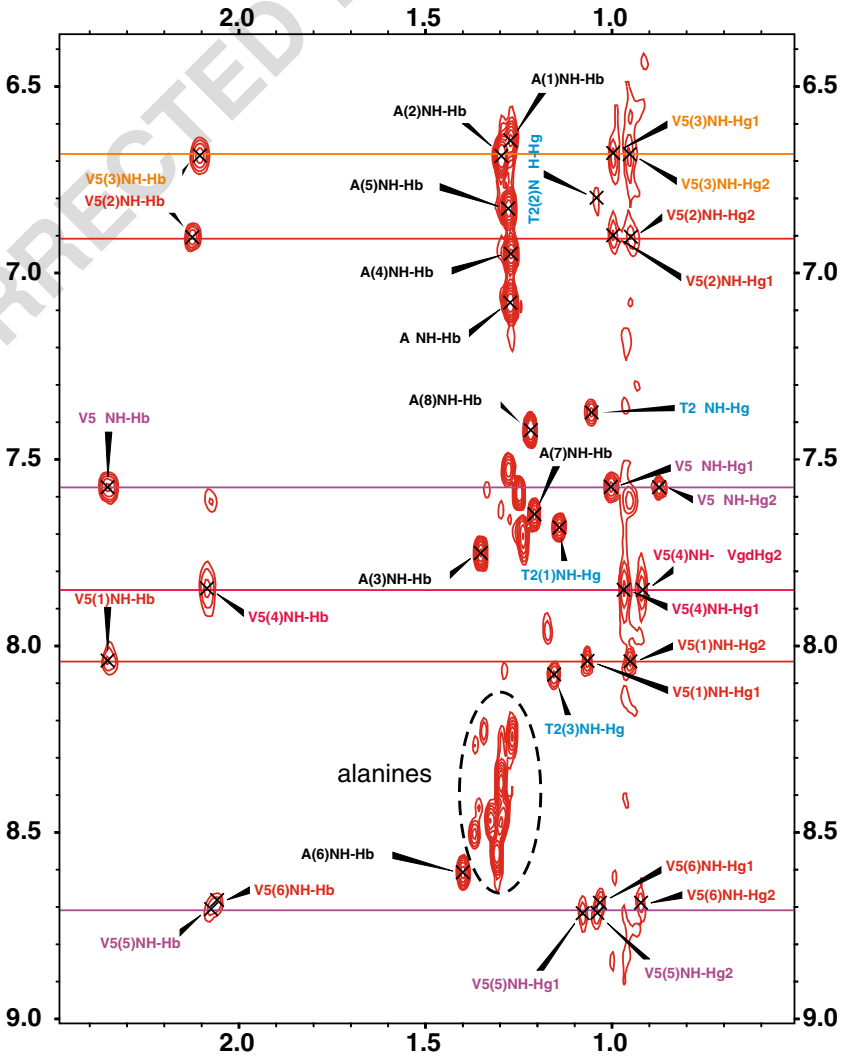

Fig. 1 Correlation of amide and sidechain protons observed in TOCSY (700 MHz, 25 °C) for alanines (A), threonine (T2), and valine (V5). Signals of different conformers are labeled with numbers in parentheses (A, A(1), A(2), etc.); the numbering order is arbitrary. Subspectra of V5 are marked with horizontal lines

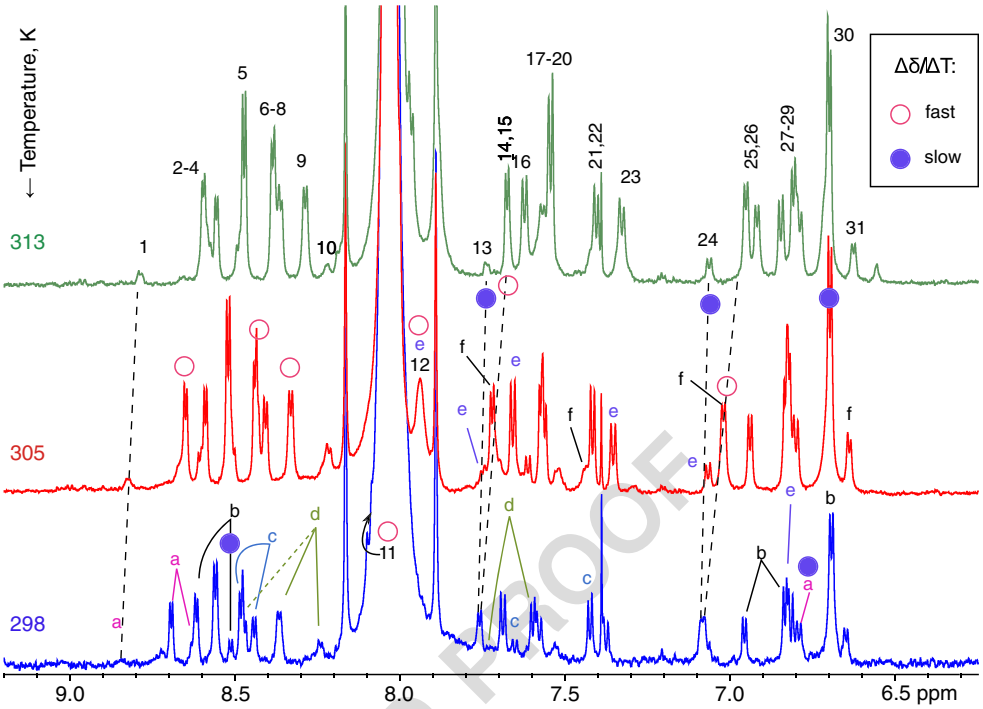

Fig. 2 Exchange-correlated NH signals of cyclosporin C in DMF and temperature dependence of the peak positions. Letters *a*–*f* stand for the groups of interconverting signals (*a*, *c*, *d*, *f*, Ala7, and Ala8; *b*, Val5; line 11, *e*, Thr2). The residual signal of DMF in all three spectra is placed at the same position for clarity

other peaks in this case are smaller and tend to have the intensity of about 27–34% or 21–24%. Six peaks have integrals ≤ 17%; these are the peaks which become more prominent at increased temperature due to an increase in their size or to the fact that they leave the overlapping region (nos. 1, 6, 10, 15, 17, and 24 belong to at least four different groups). Lines 5, 11, and 19 have no observable exchange counterparts.

Chemical shifts of alpha-protons of valine span the range of 0.4 ppm and of threonine, 0.25 ppm. Sarcosine can also be recognized by comparing TOCSY (where it has a typical signal shape) and DQF-COSY spectra with ROESY, which shows negative signals (NOE) between the geminal CH_2 protons. Several signals standing outside the crowded regions (3.4–3.8 ppm) can also be assigned to sarcosine from 1D spectra due to their typical doublet shape with a large splitting up to 16 Hz. Obtained partial assignment for alanines 7 and 8, Thr2, Val5, and Sar3 is presented in Table 1.

To characterize the observed signals in the NH resonance region, we measured their chemical shifts at different temperatures (with high-field signals of the peptide's side chains assumed fixed). Most of them were found to show moderate or fast temperature-dependent behavior [6]. Signals with $\Delta\delta/\Delta T > 6$ ppb/K are marked with empty circles, while those with $\Delta\delta/\Delta T < 3$ ppb/K are marked with filled circles. Note that "slow" signals are often met among

Q3 **Table 1** ^{1}H chemical shifts of some residues of CsC in different conformers (based on TOCSY recorded at 700 MHz, 25 °C). Low-field signal of DMF is set to 8.02 ppm. Numbering corresponds to Fig. 1

	Ala	NH	Hα	Hβ	Thr	NH	Hα	Hβ	Hγ	Val	NH	Hα	Hβ	Hγ1	Hγ2
t1.3		7.08	4.44	1.27		7.37	4.98	4.08	1.06		7.57	4.82	2.36	1.00	0.87
t1.4	1	6.64	4.53	1.27	1	7.68	4.89	4.12	1.14	1	8.04	4.65	2.35	1.07	0.95
t1.5	2	6.69	4.48	1.30	2	6.80	5.05	4.06	1.04	2	6.91	4.80	2.12	0.99	0.93
t1.6	3	7.75	4.33	1.35	3	8.08	5.14	3.98	1.16	3	6.69	5.00	2.11	1.00	0.95
t1.7	4	6.95	4.45	1.27	Sar		Hα1	Hα2		4	7.85	4.59	2.08	0.97	0.92
t1.8	5	6.83	4.47	1.28			4.92	3.81		5	8.72	4.73	2.07	1.08	1.04
t1.9	6	8.61	4.23	1.40	1		5.20	3.74		6	8.69	4.45	2.06	1.03	0.92
t1.10	7	7.65	4.93	1.12	2		5.12	3.67							
t1.11	8	7.42	4.95	1.22	3		5.10	4.40							
t1.12					4		4.71	4.09							
t1.13					5		4.74	3.53							

weak ones (6, 13, 24, 28, 29, and a component of the signal group 30), while all "fast" signals are strong. This finding correlates with the investigation of a cyclic hexapeptide by NMR and molecular dynamics reported in [7]. It was found that in chloroform, the peptide had generally more H-bonds than in DMSO, and only one H–bond was relatively stable in both media. Evidently, the situation with cyclosporin in polar media such as DMF is similar: most of amide protons are not involved in intramolecular hydrogen bonds. Conformations with additional bonded amide protons (6*b* Val; 3*a* Ala; 24*e* and 29*e* which can be assigned to Thr) also exist, but at a small population level.

In principle, chemical exchange rate can be calculated from 2D exchange spectra, but this requires measurement of both 2D integrals of diagonal and cross-peaks and corresponding signals in 1D spectra at various temperatures [8, 9]. We made a rough estimation for the exchange pair (25–31, group *f*, Ala). Very few exchange cross-peaks were observed at 15 °C in the spectrum obtained at 700 MHz; the interconversion process is frozen. The exchange rate is on the order of 0.5 s^{-1} for the process 31 → 25 at 25 °C and increases to ~1.4 s^{-1} at 37 °C, which is consistent with a high free energy barrier (on the order of 75 kJ/mol). This is most probably *cis-trans* isomerization of peptide bonds, and it is supported by multiple NOE effects observed between CHα protons (Fig. 3; note that the signals having the large splitting in the F2 axis should belong to Sar3). The leftmost signal at 5.7 ppm can be recognized as Mle9 by the cross-peak showing the *cis*-bond between Mle9 and Mle10; the same signal can be observed for cyclosporins A, C, and also B and D dissolved in chloroform (unpublished data; chemical shifts of CsB and D may be downloaded from BMRB entries 27752 and 27779). Hence, there is a conformer in DMF which resembles the major conformer existing in a polar media at least by the configuration of residues 9, 10, and the nearest sites.

3 Conclusions

Cyclosporin C in polar media behaves similarly to CsA and experiences a complex system of conformational changes. Distribution of arising conformers is nonuniform: while most of the amide ^{1}H signals have the integrals within a narrow range, approximately one-quarter of them has a reduced intensity. Lesser number of signals shows a slow temperature dependence, which means that intramolecular hydrogen bonds of the peptide are mostly disrupted and point into the solvent. The fraction of the molecules having an increased number of intramolecular H-bonds is small and corresponds to the mentioned minor NH signals. Weakening of the bond $C_5=O \leftarrow H–N_2$ and disruption of the β-sheet was supposed in [10] according to FT-VCD optical studies. This finding is supported by our data since

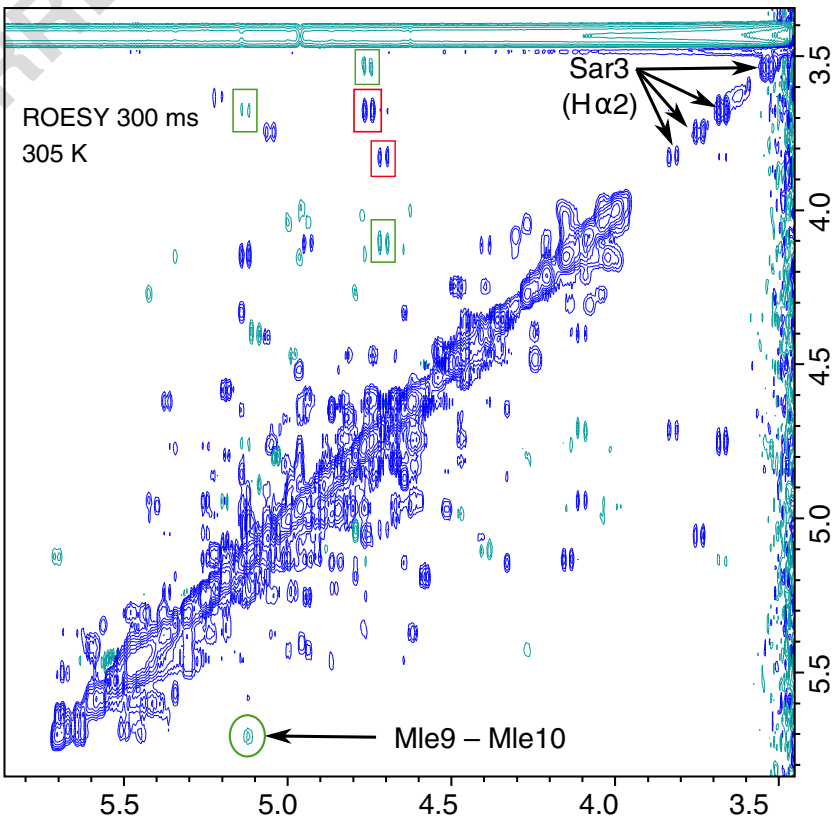

Fig. 3 Part of 2D ROESY spectrum of CsC in DMF obtained at 700 MHz, 32 °C, with the mixing time of 300 ms. Both NOE and exchange peaks are observed (NOE peaks are green; exchange, blue). The NOE between Hα protons of Mle9 and Mle10 is shown with an arrow; this cross-peak is also seen for CsC and CsA in chloroform. Some signals of Sar3 are also shown: NOEs in pairs Hα1–Hα2 (green squares) and exchange between different conformers (red squares)

the mentioned signal has several other exchange counterparts (group *e*) having somewhat larger $\Delta\delta/\Delta T$ values (peak 23 with $\Delta\delta/\Delta T = 4.4$ ppb/K; peak 11 with 7.8 ppb/K), and still, residues 2 and 5 are those which show amide protons which remain hydrogen-bonded in some of the conformers.

Information on transformation rates and energy barriers may be useful for carrying out MD simulations. Increasing temperature of the system and decreasing the force constants for peptide bonds (angle ω) were necessary in [4] to cover the accessible conformational space in a reasonable time (trajectory duration was 250 ns). It would be helpful in simulations of this kind to know the number and fraction of conformers and their spectroscopic peculiarities (large down- or up-field shifts of NH resonances and involvement of atoms in H-bonds) to compare simulation results with an experiment.

Funding information This work was supported by the Russian Science Foundation (project no. 18-73-10088).

References

1. Craik, D. J. (2006). Seamless proteins tie up their loose ends. *Science, 311*, 1563–1564. https://doi.org/10.1126/science.1125248.
2. Lee, D. L., & Hodges, R. S. (2003). Structure activity relationships of de novo designed cyclic antimicrobial peptides based on gramicidin S. *Biopolymers, 71*, 28–48. https://doi.org/10.1002/bip.10374.
3. Cirac, A. D., Torné, M., Badosa, E., Montesinos, E., Salvador, P., Feliu, L., & Planas, M. (2017). Rational design of cyclic antimicrobial peptides based on BPC194 and BPC198. *Molecules, 22*, 1054. https://doi.org/10.3390/molecules22071054.
4. Wang, C. K., Swedberg, J. E., Harvey, P. J., Kaas, Q., & Craik, D. J. (2018). Conformational flexibility is a determinant of permeability for cyclosporin. *The Journal of Physical Chemistry. B, 122*, 2261–2276. https://doi.org/10.1021/acs.jpcb.7b12419.
5. Damaso, C. R., & Moussatch, N. (1998). Inhibition of vaccinia virus replication by cyclosporin A analogues correlates with their affinity for cellular cyclophilins. *The Journal of General Virology, 79*, 339–346. https://doi.org/10.1099/0022-1317-79-2-339.
6. Kessler, H. (1982). Conformation and biological activity of cyclic peptides. *Angewandte Chemie, International Edition, 21*, 512–523. https://doi.org/10.1002/anie.198205121.
7. Farley, K. A., Ye, C., Navarro-Vázquez, A., Limberakis, C., Anderson, D., Yan, J., Shapiro, M., Shanmugasundaram, V., & Gil, R. R. (2019). Cyclic peptide design guided by residual dipolar couplings, J-couplings, and intramolecular hydrogen bond analysis. *The Journal of Organic Chemistry, 84*(8), 4803–4813. https://doi.org/10.1021/acs.joc.8b02811.
8. Efimov, S., Zgadzay, Y., & Klochkov, V. (2014). Observation of conformational exchange in cyclosporin in media of varying polarity by NMR spectroscopy. *Applied Magnetic Resonance, 45*, 1225–1235 s00723-014-0602-y.
9. Perrin, C. L., & Dwyer, T. J. (1990). Application of two-dimensional NMR to kinetics of chemical exchange. *Chemical Reviews, 90*, 935–967. https://doi.org/10.1021/cr00104a002.
10. Bodack, L. A., Freedman, T. B., Chowdhry, B. Z., & Nafie, L. A. (2004). Solution conformations of cyclosporins and magnesium-cyclosporin complexes determined by vibrational circular dichroism. *Biopolymers, 73*, 161–177. https://doi.org/10.1002/bip.10513.