# Cis-Trans Isomerization in Cyclosporin C Dissolved in Acetonitrile

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**Abstract**

Cyclosporin is an 11-amino acid cyclic peptide with pharmacologically valuable properties which has a variety of actual and potential applications. Its activity relies on the cell membrane permeability which, in turn, depends on the structure of cyclosporine and its ability to change the conformation. In this work, conformational exchange processes occurring in cyclosporin C were studied using one- and two-dimensional nuclear magnetic resonance spectroscopy. The free energy barrier separating two major conformers observed in polar solution (acetonitrile) was found to be 77 ± 2 kJ/mol. Less populated conformation states are also present in the solution, which agrees with the ease of formation of multiple forms revealed by MD simulations of cyclosporin C.

*Keywords:* Cyclosporin, NMR, cis-trans isomerization, chemical exchange, EXSY

## 1. Introduction

Cyclosporin A (CsA) is well known for its immunosuppressive activity and it is widely used in immunosuppressive therapy in organ transplantation [1]. Cyclosporins also have antiparasitic, antiviral, antifungal and anti-inflammatory properties, so they can be utilized in the treatment, for instance, of hepatitis B, dry eye disease or autoimmune diseases [2–5]. The pharmaceutical activity of cyclosporin depends on its membrane permeability. High membrane perme-

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ability increases the bioavailability of drugs, whose target molecules are found in cell cytoplasm, and allows oral administration. However, some membrane-impermeable derivatives of CsA have purposefully been developed in order to make them able to specifically interact with extracellular cyclophilin [6, 7].

Cyclosporins demonstrate conformational flexibility, and different molecular structures usually coexist in polar solvents. Thus, when administered inside, cyclosporin is distributed into a number of conformers containing both required and unneeded forms; these different forms have different properties and activity. Thus, for the possibility of increasing drug efficacy and maximizing the concentration of the required conformer, it is necessary to learn more about how structure of cyclosporins changes and distribution into diverse conformers occurs.

There are hundreds of analogues of CsA and most of them differ from CsA in one or two sites by a change of amino acid or N-methylation state. For example, the aminobutyric acid (Abu2), which is located at the position 2 in CsA is replaced with threonine (The2) in cyclosporin C (CsC). Regarding the drug efficacy, CsA is considered to be the most effective, but administration of it is often accompanied by side effects such as nephrotoxicity, hypertension, hyperlipidemia, hypertrichosis, neurotoxicity and hepatotoxicity [8–10]. Some CsA analogues can also be immunosuppressants but with weaker side effects [11, 12]. Experiments with CsC have shown that it has a strong immunosuppressive property while it remains less nephrotoxic than CsA [11].

Thus, arising of multiple conformations and factors influencing this phenomenon are important questions when considering cyclosporins or cyclic peptides in general. The aim of this work was to observe the conformational behavior of CsC (Figure 1) in deuterated acetonitrile ($CD_3CN$) by NMR spectroscopy and to determine the parameters of conformational exchange basing on the theory described in [13].

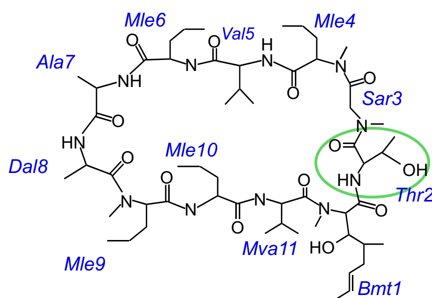


Figure 1: Chemical structure of cyclosporin C.

## 2. Materials and Methods

NMR measurements were carried out on a Bruker Avance III HD 700 spectrometer (Center of Shared Facilities of Kazan Federal University). Solution was prepared in deuterated acetonitrile ($CD_3CN$), the sample concentration was 1.4 mM (for all conformers). Two-dimensional spectra (DQF-COSY, TOCSY, ROESY, HSQC and HMBC) were recorded at the temperature of 25°C. Exchange spectroscopy (EXSY) spectra were obtained at different temperatures from 12 to 30°C, the mixing times were in the range from 0.15 to 0.35 s. A series of one-dimensional $^1$H NMR spectra was taken at the temperatures from 12 to 38°C in the spectral window of 12 ppm and spectral resolution of 0.257 Hz per data point.

Processing was carried out with the aid of the TopSpin software package. A combination of 2D spectra were used for signal assignment. Peak integration in EXSY spectra was made using the Sparky program [14].

## 3. Results

Signal assignment of atoms in the backbone and side chains was achieved using the set of 2D spectra (DQF-COSY, TOCSY, ROESY, HSQC and HMBC). Total signal assignment was obtained for the major conformer of CsC. Another set of 11 $\alpha$-CH peaks with weaker intensity was also observed in the HSQC spectrum, which allowed identification of the second conformer.

Conformational exchange processes were studied with the aid of 2D EXSY spectra recorded at different temperatures from 12 to 30°C. Four pairs of signals were selected for further analysis of chemical exchange: threonine Thr2 NH – Thr2* NH, valine Val5 NH – Val5* NH, alanine Ala7 NH – Ala7* NH, and leucine Mle9 H$\alpha$ – Mle9* H$\alpha$. They were placed in a relatively free regions of the spectra, far from signal crowding, and thus could be integrated.

Signals of amide protons experience downfield changes in chemical shifts with increasing temperature with different rates (Table 1). Signals from Val5 NH and Dal8 NH overlapped at 12°C, and hence integration in 1D spectra was impossible and parameters of conformational exchange were not calculated for the pair Val5 NH – Val5* NH at this temperature. Exchange processes expectedly slow down at low temperatures, so that cross-peaks in 2D spectra were very weak and could be integrated with a limited accuracy.

Table 1: $^{1}$H NMR chemical shifts of selected residues of CsC in major and minor conformers (based on EXSY recorded at 700 MHz). Numbering of signals corresponds to Fig. 1. Asterisk (*) at the name of amino acid indicates whether this amino acid belongs to the second conformer

| $t$, °C<br>atom | 12 | 17 | 21 | 26 | 30 | 34 | 38 | $\Delta\delta/\Delta T$,<br>ppb/K |
|---|---|---|---|---|---|---|---|---|
| Thr2 NH | 7.854 | 7.831 | 7.810 | 7.790 | 7.772 | 7.756 | 7.740 | −4.38 |
| Thr2* NH | 7.060 | 7.058 | 7.070 | 7.079 | 7.085 | 7.092 | 7.098 | 1.62 |
| Val5 NH | | 7.328 | 7.325 | 7.321 | 7.319 | 7.315 | 7.311 | −0.81 |
| Val5* NH | 6.726 | 6.737 | 6.717 | 6.696 | 6.680 | 6.665 | 6.650 | −4.11 |
| Ala7 NH | 7.535 | 7.516 | 7.501 | 7.482 | 7.467 | 7.452 | 7.437 | −3.77 |
| Ala7* NH | 6.431 | 6.423 | 6.417 | 6.412 | 6.408 | 6.403 | 6.399 | −1.20 |
| Mle9 H$\alpha$ | 5.650 | 5.656 | 5.663 | 5.669 | 5.674 | 5.679 | 5.684 | 1.34 |
| Mle9* H$\alpha$ | 5.407 | 5.409 | 5.414 | 5.416 | 5.419 | 5.422 | 5.424 | 0.68 |

The most significant difference in chemical shifts for the same amide proton in different conformers was 1.07 ppm for the pair Ala7 NH – Ala7* NH at 26°C. As the temperature rises, this difference reduces slightly for pairs Ala7 NH – Ala7* NH and Thr2 NH – Thr2* NH (from 1.104 to 1.038 ppm and from 0.794 to 0.642 ppm respectively) but it increases for pairs Val5 NH – Val5* NH and

Mle9 H$\alpha$ – Mle9* H$\alpha$ (from 0.591 to 0.661 ppm and from 0.243 to 0.26 ppm, respectively). A large variation of the chemical shift (in the order of 1 ppm) for a certain atom in different forms of the molecule should represent a drastic difference in the local magnetic environment. We can expect thus that alanine Ala7 and threonine Thr2 are close to the place where cis-trans isomerization of a peptide bond occurs. This may be either the adjacent amino acid residue or a more distant segment of the peptide chain which turns out to be close in space due to oblate shape of the cyclosporin's ring.

Values o f$\Delta\delta/\Delta T$ more negative than $-4.5$ ppb/K are assumed with hydrogen bonding of the corresponding amide proton NMR signal [15]. This method has a limited accuracy, but still allows to discriminate two main conformers in CsC according to the behavior of their amide protons. Thus, the major form is expected to have intramolecular H-bonds in positions of residues Thr2 and Ala7, while the other form (marked with asterisk in Table 1) has these bonds solvent-exposed and Val5*, on the contrary, is hydrogen-bonded.

To calculate the rates of conversion of the major (A) and minor (B) conformers to each other and parameters of conformational exchange, one needs to measure integrals of two diagonal peaks $I_{AA}$ and $I_{BB}$ (signals of the exchanging conformers) and two cross-peaks $I_{AB}$ and $I_{BA}$ from two-dimensional EXSY spectra, as well as corresponding signals in one-dimensional spectra [13, 16]. This conversion is characterized by rate constants $k_{AB}$ ($A \to B$), $k_{BA}$ ($B \to A$) and the summary exchange rate $k = k_{AB}+k_{BA}$. In the case of two-site exchange the rate constant $k$ can be found from Equation (1) [13]:

$$k = \frac{1}{t_m} \ln \frac{r+1}{r-1}\,, r = 4X_A X_B \frac{I_{AA}+I_{BB}}{I_{AB}+I_{BA}} - (X_A - X_B)^2\ , \tag{1}$$

where $t_m$ is the mixing time; $X_A$, $X_B$ are molar fractions of the conformers (in our case, major and minor), $X_A + X_B = 1$. The mole fractions $X_A$ and $X_B$ were determined by integration of one-dimensional spectra at temperatures 12, 26, 34 and 38°C. Relative integral intensity $X_B/X_A$ allows obtaining the energy difference between the conformers $\Delta G_0$:

$$\Delta G_0 = -RT \ln(X_B/X_A)\,, \tag{2}$$

where $R$ is the universal gas constant and $T$ is the absolute temperature.

The rate constants $k$, $k_{AB}$, $k_{BA}$, and the energy difference $\Delta G_0$ were calculated for the temperatures 12, 26, 34 and 38°C, and then the average value of $\Delta G_0$ for each considered signal pair was used to interpolate the values of $X_A$, $X_B$, $k$, $k_{AB}$, $k_{BA}$ at 17, 21, and 30°C (1D spectra were not obtained at these temperatures). The linear form of the Eyring equation was used to determine activation enthalpy $\Delta H^{\neq}$, activation entropy $\Delta S^{\neq}$, and activation energy $\Delta G^{\neq}$ (free energy barrier):

$$\ln \frac{k}{T} = -\frac{\Delta H^{\neq}}{RT} + \ln \frac{k_B}{h} + \frac{\Delta S^{\neq}}{R}\,, \tag{3}$$

where $k_B$ is the Boltzmann constant and $h$ is the Planck constant. Graph of the dependence of $\ln(k/T)$ on $1/T$ produces a straight line: the activation enthalpy $\Delta H^{\neq}$ can be calculated from the slope of this line, and the activation entropy $\Delta S^{\neq}$ can be determined from the $y$-intercept (Figure 2). Equation $\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$ allows calculating the free energy barrier $\Delta G^{\neq}$. These calculations were performed for each selected signal of four selected residues. The results are presented in Table 2.

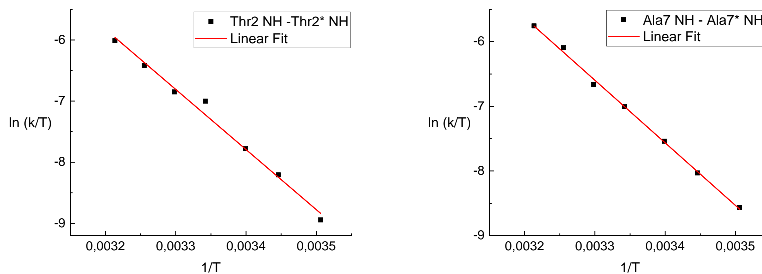


Figure 2: Example plots of the dependences of $\ln(k/T)$ on $1/T$.

The exchange rate constants increase significantly as the temperature rises. For instance, the rate of conversion from the main to minor conformer $k_{AB}$ grows substantially for threonine Thr2 NH and alanine Ala7 NH: for Thr2 NH the ratio $k_{AB}(38°\mathrm{C})/k_{AB}(12°\mathrm{C})$ is 24.6, and the ratio $k_{BA}(38°\mathrm{C})/k_{BA}(12°\mathrm{C})$ is 19.7; for Ala7 NH the rate constant $k_{AB}$ rises 22.2 times and $k_{BA}$ increases

17.6 times in the same temperature range. Values of $k_{AB}$ and $k_{BA}$ are of the same order of magnitude for the residue Mle9. The value $k_{BA}$ is about 10 times higher than $k_{AB}$ in the analyzed amide proton signals, representing the fact that the major conformer A is populated much more than the conformer B, and the energy difference between the conformers $\Delta G_0$ is relatively high. However, for leucine Mle9 this energy difference 5–6 times smaller. The activation energy of the minor form $\Delta G^{\neq}$ of all analyzed residues is approximately the same and the average value is about 77.3 kJ/mol.

Table 2: Chemical exchange rates ($s^{-1}$) and parameters of conformational exchange (kJ/mol) of CsC for the selected signals (energy difference between the conformers at 26°C and activation energy are given at 26°C). Exchange rates for Val5 NH – Val5* NH were calculated at temperatures 17 and 38° instead of 12 and 38°C

| | 12° | | 38° | | $\Delta G_0$ | $\Delta H^{\neq}$ | $\Delta G^{\neq}$ |
|---|---|---|---|---|---|---|---|
| | $k$ | $k_{AB}/k_{BA}$ | $k$ | $k_{AB}/k_{BA}$ | | | |
| Thr2 NH | 0.037 | 0.054/0.032 | 0.762 | 0.133/0.63 | 4.2 | 81.7 | 77.1 |
| Ala7 NH | 0.054 | 0.0054/0.049 | 0.985 | 0.12/0.866 | 5.2 | 80.5 | 76.5 |
| Mle9 H$\alpha$ | 0.014 | 0.0056/0.008 | 0.365 | 0.155/0.21 | 0.82 | 91.5 | 79.1 |
| Val5 NH | 0.083 | 0.0081/0.075 | 1.06 | 0.1/0.955 | 5.4 | 84.5 | 76.3 |

## 4. Discussion

The conformational exchange process in cyclosporin C was investigated by analysis of selected NMR signals of atoms Thr2 NH, Val5 NH, Ala7 NH, and Mle9 H$\alpha$ in acetonitrile ($C_2D_3N$, $\epsilon = 37.5$). A prominent distinction in chemical shifts of the amide protons in two observed conformers (e.g., 1.07 ppm for Ala7 NH at 26°C) was found. As the temperature rises, the exchange rate determined from EXSY spectra increases. This allows finding the free energy barrier between the two states: $77 \pm 2$ kJ/mol. The energy difference between the stable conformations found from 1D spectra was approximately $8 \cdot 10^2$ J/mol for Mle9 H$\alpha$ signal and $(4.2\text{–}5.4) \cdot 10^3$ J/mol for NH signals. These findings are indicative of *cis-trans* isomerization occurring in one or several sites of the peptide backbone.

Kinetic properties of cyclosporin A dissolved in tetrahydrofuran (THF, $C_4D_8O$, $\epsilon = 7.6$) and in LiCl-THF were examined in [17] by NMR spectroscopy. It was found that CsA in THF had a single form with a Mle9–Mle10 *cis* peptide bond, but CsA in LiCl–THF had the Mle9–Mle10 bond in the *trans* configuration. The barrier energy of *cis-trans* isomerization of this peptide bond is about 19 kcal/mol (79.5 kJ/mol). In [18] high-performance liquid chromatography (HPLC) was used to study properties of the conversion of cyclosporins A, C and D which were dissolved in an acetonitrile–water mixture. The free energy barriers of conformational conversions were 17–18, 16 and 18 kcal/mol for CsA, CsC and CsD, respectively.

In agreement with these observations is the value of activation energy of $81 \pm 2$ kJ/mol found for cyclosporin A in chloroform in [19]. An earlier investigation of the backbone dynamics of CsC in dimethylformamide ($(CD_3)_2NCDO$, $\epsilon = 36.7$) by NMR spectroscopy allowed us to calculate $\Delta G^{\neq} = 75$ kJ/mol, which was also attributed to *cis-trans* isomerization of peptide bonds [20]. The difference between different solvents tested in the studies of cyclosporins is the number of conformers and their relative population. In polar media several stable conformers with similar energies can exists simultaneously; the most clearly this fact is manifested, for example, in dimethyl sulfoxide or DMF. In other solvents such as methanol or acetonitrile the number of conformers turns out to be smaller; moreover, their relative population is sensitive to the presence of ions. Finally, in solvents which have low polarity and do not form hydrogen bonds, a single conformation is present.

It would be interesting to investigate in details structural properties of individual conformers. NMR has the best possibilities to solve this problem but suffers from signal overlap when the number of coexisting molecule forms is too large. For instance, six conformers in case of cyclosporin produce a set of signals similar to that expected from a 66-member protein, and the signal assignment methods based on 2D spectroscopy will fail in this case. Seeking for media in which the number of prevalent forms is limited to two or three makes possible studies of individual conformers of exchanging molecules such as cyclosporin.

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