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Complexation and medium effects on the conformation of cyclosporin A studied by NMR spectroscopy and molecular dynamics calculations

Cyclosporin A (CSA*) is an important immunosuppressive drug [1–3] widely used clinically to prevent graft rejection in organ transplantations. A detailed knowledge of the structure in solution under different environmental conditions is the basis for structure–activity relationships that allow the design of new derivatives with higher activity and fewer side-effects. Some years ago we investigated CSA in solvents of low polarity ($CDCl_3$ and C_6D_6) by NMR methods [4–6]. The structure of CSA in these solvents was derived from these measurements and compared to the X-ray structure [7].

Restraint molecular dynamics (MD) calculations under different conditions (*in vacuo*, in crystal, in H_2O, and in CCl_4) [8–10] were performed to study the influence of the environment on the conformation.

As the methodology for structure determination in solution by NMR spectroscopy has improved dramatically in the last few years, it seemed reasonable to study this molecule by state of the art techniques again. In addition, we investigated the structure of CSA in THF-d_8 solution and the influence of complexation by lithium salts.

Reinvestigation of cyclosporin A in $CDCl_3$

Our first study was performed in the positive nuclear Overhauser enhancement (NOE) region (rapid motion limit) at room temperature in $CDCl_3$, a solvent of low viscosity [5–7]. Under these conditions it was only possible to get ranges of distances with low accuracy. In total, 58 distance restraints were used in our first structure determination.

However, cross-relaxation rates, which are functions of interproton distances, can best be determined by measurement of build-up rates in the slow motion limit (negative NOE effects) [11]. Hence, we measured build-up rates of cyclosporin A in $CDCl_3$ solution at 600 MHz. The higher spectrometer frequency used in this study is advantageous compared to the previously used 300 MHz spectra: in addition to the higher resolution and higher sensitivity, the shift into the direction of slow motion is important. As NOE effects of CSA are small at room temperature in $CDCl_3$ at 600 MHz, the measuring temperature was lowered to 250°K.

NOESY spectra with mixing times of 80, 120, 160, 200, 240 and 300 msec were recorded as well as the build-up of the NOE effects in the usual procedure.†

In total we got more than 500 cross-peaks in the NOESY spectrum. We found that the distances determined here, using the two-spin-approximation, agreed very well with the upper and lower boundaries derived from our previous steady-state NOE effects. However, it has been shown and discussed at length in the literature that the interconversion of cross-relaxation rates into distances using the two-spin-approximation may result in significant systematic deviation from actual distances, especially for longer distances [12]. We therefore applied the iterative relaxation matrix approach (IRMA) to determine the structure [13–15]. Although these calculations are not yet finished, the results obtained so far are in complete agreement with our previous assignments in CSA as well as with the structure of CSA.

Some of the NOE cross-peaks could not be assigned unambiguously to a proton-proton NOE as, in addition to NOE, chemical exchange with the minor populated conformation (population approx. 6%) is also possible. Those cross-peaks were omitted. Also, the cross-peaks on both sides of the diagonal could not be evaluated in every case. Hence, the number of reliable distance data was reduced to 191 (Table 1).

Using the IRMA procedure in connection to the GROMOS 87 MD package [16], we obtained a trajectory (mean over 15 psec) which was almost identical in the conformational angles of the backbone. However, in agreement with our previous observations we found larger deviations in the side-chain conformations. Although stereochemical assignments were used, some side chains were not found in those conformations which unambiguously follow the J coupling constants [7, 17]. This supports our general strategy for peptide conformational analysis: at least χ_1 of the side chain is derived from homo- and heteronuclear coupling constants [17] and kept fixed in the MD calculation in the most populated conformation [18].

* CSA, cyclosporin A; THF, tetrahydrofuran; MD, molecular dynamics; and NOE, nuclear Overhauser enhancement.

† The NMR program of Prof. Robert Kaptein, Utrecht, was used on a VAX 6210 computer.

Table 1. NOE effects obtained for cyclosporin A in $CDCl_3$, in THF, and in THF with addition of LiCl

	$CDCl_3$	THF	Li-complex
Frequency	600 MHz	500 MHz	500 MHz
Temperature	250°K	250°K	300°K
Integrated NOE cross-peaks	Approx. 500	375	359
Assigned NOE effects	191	77	74
Intraresidue NOE effects			
NH-alpha	4	4	3
NH-beta	5	4	3
Alpha-beta	14	6	9
NMe-alpha	5	2	2
NMe-beta	7	4	5
Others	57	18	23
Interresidue NOE effects			
Sequence	52	25	26
Long range	47	16	3

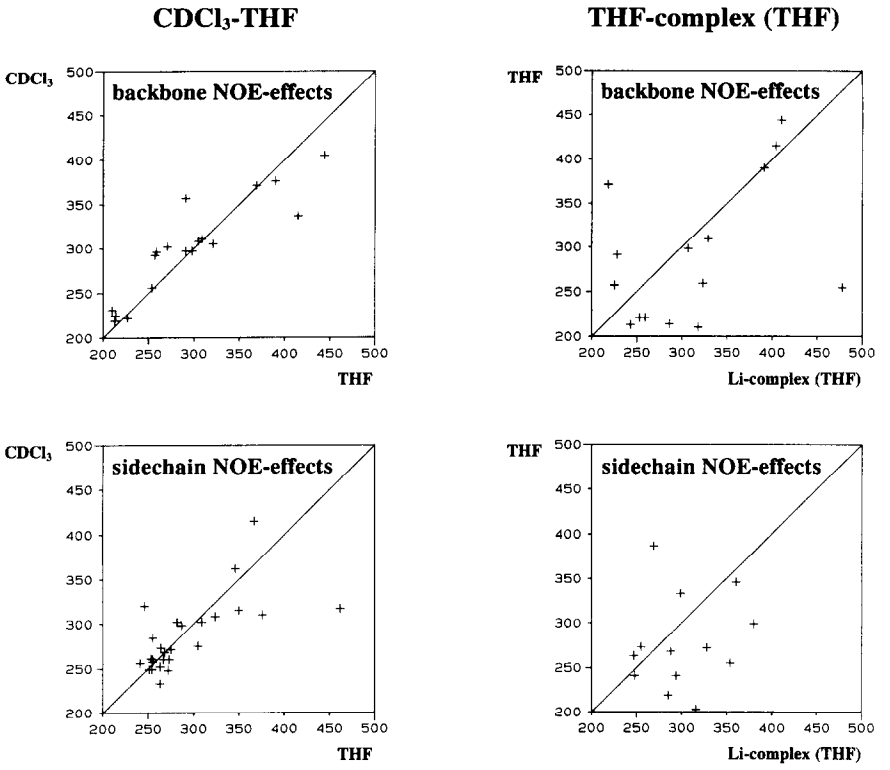

Fig. 1. Six hundred MHz ^{1}H NMR spectra of cyclosporin A in $CDCl_3$, THF-d_8 and in THF-d_8 with addition of 30.9 equivalent LiCl.

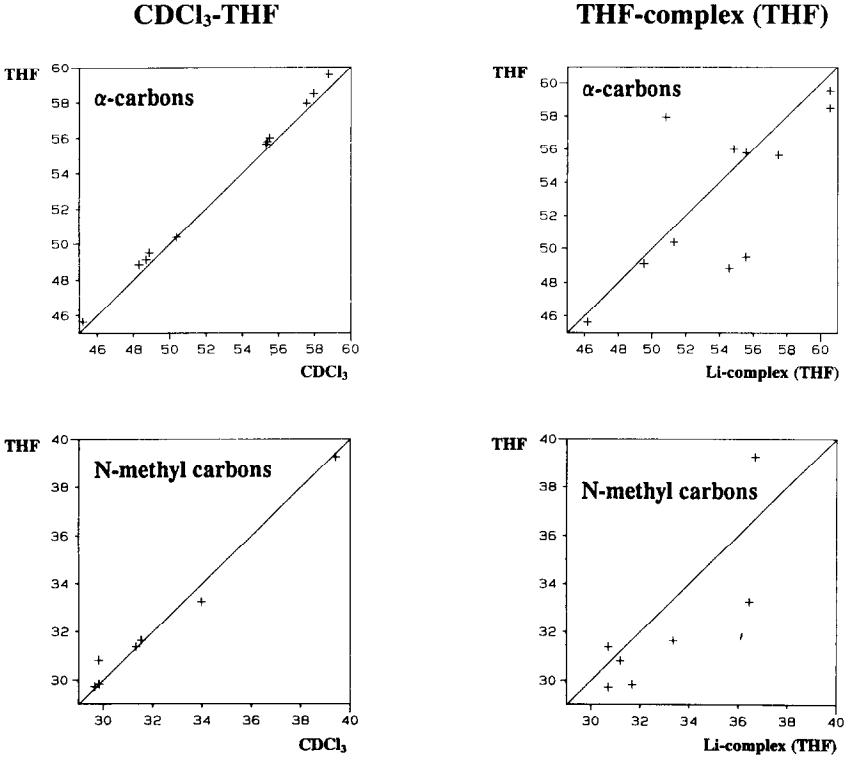

Fig. 2. Cross-sections of C_α of MeLeu^{10} and $C_{\gamma 2}$ of Val^5 of the 250 MHz TOCSY-DEPT spectrum of CSA in THF-d_8. For a comparison, the analogue TOCSY-INEPT spectrum is shown. The total spin systems of the amino acid proton spin system of MeLeu^{10} and Val^5 can be assigned on the chemical shift of the two carbons.

Structure of cyclosporin A in THF-d_8 and its lithium complex

The solubility of CSA in water is too low for a quantitative conformational analysis. In dimethyl sulfoxide (DMSO) several conformations are observable in equilibrium slow on the NMR time scale. Hence, it was difficult to find other solvents to study the influence of the environment on the conformation of CSA. One solvent, which was used recently in the solubilization of peptides by the addition of lithium-salts, is THF [19, 20]. In fact, CSA was very soluble in this solvent and exhibited also only one dominating conformation (see Fig. 1), as in $CDCl_3$ and C_6D_6. However, the 1D-NMR spectrum in THF looked different from those in the other solvents. Hence, it had to be determined whether the conformation was changed or if the different appearance was just a solvent effect on chemical shifts. A detailed study using several homo- and heteronuclear 2D-NMR techniques [21] was performed to assign the signals to the chemical constitution. Among these techniques there is a new variant of a heteronuclear correlation, the TOCSY-DEPT, which was introduced recently by us [22] and which was especially helpful in the analysis of the crowded regions. The TOCSY-DEPT uses proton excitation and labelling in t_1 followed by a homonuclear TOCSY transfer, which mixes the proton spin systems. The transfer to carbon is by DEPT, which has several advantages [22] over the previously suggested INEPT transfer for this purpose [23]. A section at two carbons (C_α of MeLeu^{10} and $C_{\gamma 2}$ of Val^5, Fig. 2) showed that in such a spectrum both the proton and carbon signals can be assigned unambiguously. Sequence analysis and quaternary carbon assignment followed similar procedures as used for the $CDCl_3$ solution [5, 6] but here only proton-detected (= inverse) techniques [21] were used.

Having the assignments, NOE effects were measured at 250°K (500 MHz) (Table 1). The same procedure was used for the lithium complex (Fig. 1 and Table 1); however, NOE could be determined at 300°K, as the slower tumbling of the complex shifted the cross-relaxation into the negative NOE region.

Although a detailed conformational analysis is not yet finished, the comparison of chemical shifts in the different environments yielded interesting results. As can be seen from Fig. 3, the chemical shift values of all α-carbons and the N-methyl carbons correspond completely to each other in the $CDCl_3$ or THF solvent. As carbon chemical shift values are strongly determined by the conformation [24, 25], it is obvious that no conformational change was induced by changing the solvent from $CDCl_3$ to THF. Similar conclusions can be drawn from the NOE effects (Fig. 4); however, the correlation was not as high as for the carbon chemical shifts.

If one compares the effect of the addition of lithium chloride in the same manner, the difference is evident. Chemical shifts and NOE derived distances were drastically changed by the addition of lithium salt, indicating that the conformation of the complex is not identical to that of the uncomplexed CSA.

Molecular dynamics calculations of CSA in different environments

As pointed out above, in solvents of higher polarity there are changes in the conformation that are directly visible in the NMR spectra by appearance of more conformations in equilibrium slow on the NMR time scale. Such systems are too complex to study by NMR spectroscopy with high accuracy. Hence, we performed MD calculations simulating the conformations in H_2O and CCl_4 and compared these results with previously performed calculations of CSA in the crystal and as an isolated molecule. For water a simple rigid three-point charge model was used, whereas the unpolar solvent was treated as a united atom without coulombic interactions, as a pure Lennard–Jones liquid [10]. The calculations were started from the X-ray structure

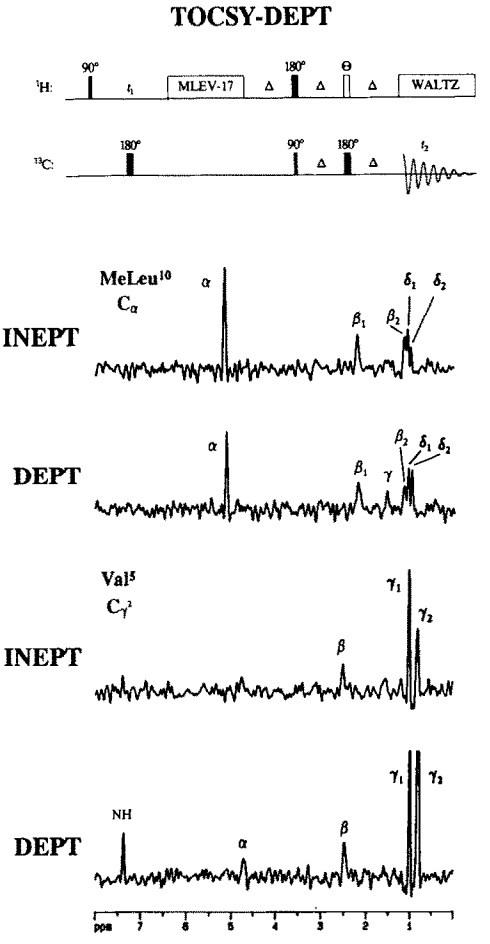

Fig. 3. Comparison of the chemical shifts of the α-carbons and the N-methyl carbons of CSA in $CDCl_3$, THF (left) and THF without and with lithium salt addition (right).

of CSA or the solvent structure calculated from restrained MD with the isolated molecule [8]. The details of the calculations are given elsewhere [10]. However, several features of these calculations should be pointed out here: the structures calculated in the Lennard–Jones solvent (without restraints!) do not deviate far from the initial restraint MD structure of the isolated molecule. Hence, the assumption that the restraints obtained from the NMR experiments are used to mimic the solvent environments in an *in vacuo* calculation seems to be justified.

It is interesting to note that the side chain of MeBmt sticks out in the Lennard–Jones solvent, whereas it is always folded back in *in vacuo* calculations without fixation in the conformation obtained from $^3J_{HH}$ coupling constants and IR measurements [7]. This proves our initial assumption that the tendency to optimize van der Waals' interaction leads to a maximal folded structure in *in vacuo* calculations. Similar observations have been made in other cyclic peptides (see, for example, Ref. 18). If we now calculate CSA in a water environment, the MeBmt side chain is folded back again as it is in the crystal and *in vacuo*. Now the polarity of water squeezes together the lipophilic molecule.

Hydrogen bonds are not explicitly included in the GROMOS MD program, but are the result of the calculation if one defines a hydrogen bond according to some

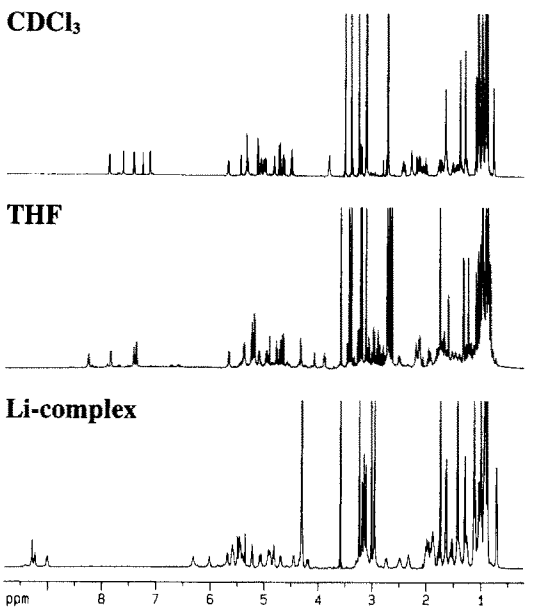

Fig. 4. Comparison of the NOE-derived distances in CSA in different environments: $CDCl_3$/THF and THF without and with addition of lithium salt.

special criteria (distances and angles). In the unpolar solvent the hydrogen bond pattern found in the isolated molecule is generally retained. However, the two hydrogen bonds in the β-pleated sheet (Abu^2-CO···HN-Val^5 and MeVal^{11}-CO···HN-Ala^7) show a lower occupancy (20–30%). On the other hand, the two γ-turns between MeVal^{11}-CO···HN-Abu^2 and Val^5-CO···HN-Ala^7 show a higher occupancy in the Lennard–Jones solvent. The two halves of the ring are even stronger bent about the axis through the C_α-atoms of MeBmt^1 and MeLeu^6.

In water these two γ-bents are almost completely gone. Also, only two NH-groups (Abu^2, D-Ala^8) are involved in hydrogen bonds, whereas all carbonyls form hydrogen bonds to water hydrogens.

However, we want to point out that the structure in water will certainly not resemble the "true" conformational space of CSA. The calculations started from the X-ray structure and conformational changes such as *cis-trans* isomerism about peptide bonds cannot occur in the time scale of the calculations. However, those interconversions are observed already in DMSO and are expected to occur also in water. Hence, the structure of CSA in water expresses probably only the first moment when the molecule leaves a lipophilic medium and enters the hydrophilic cytosol. As this moment is important in the recognition by receptors, such as antibodies, the calculated structure in water may have some biological relevance.

It is important to note that the calculations of CSA embedded in solvent molecules lead to a much slower approach to the equilibrium conformation. This is expressed in the autocorrelation functions [8] which show a relaxation time of less than 1 psec for the crystal and *in vacuo* calculation but more than 4 psec in water or high mass Lennard–Jones solvent [10]. A faster approach of the equilibrium can be achieved in the latter case by an artificial reduction of the mass of the solvent molecule. However, one has to keep in mind that any calculation which includes the solvent needs a longer time to reach equilibrium as the viscosity of the solvent slows down the mobility of the solute. Hence, a longer calculation time span for the determination of the mean trajectory is also advised. This, however, increases the cost of the already extensive calculation even more.

In summary, the conformation of cyclosporin A was investigated in different environments. Whereas there was no distinct difference between the structure of CSA in $CDCl_3$ and THF, the addition of LiCl caused a conformational change. Also, using polar solvents (DMSO, water) induced conformational changes in the backbone as well as the side chains.

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Structures from NMR distance constraints

Although advances in NMR have elevated structural determination of peptides and small proteins in solution to the level of a routine tool [1], the nature of the structural information obtained is not often appreciated. The best known method for structural analysis, X-ray crystallography, yields results in Cartesian coordinate space—the average position of each heavy atom in a molecule. In contrast, NMR studies reveal information in distance and torsional space. Distances between pairs of atoms are determined from nuclear Overhauser effects (NOEs) [2], paramagnetic enhancement of relaxation [3], or hydrogen bonding. Torsion angle constraints are derived from coupling constants and Karplus-type relationships [4]. Like X-ray, these reflect the ensemble of conformations present during the time frame of the measurement [5]. Unlike X-ray, however, these observables can be measured even in regions of structure that are highly disordered and have no characteristic conformation in solution. Generation of atomic coordinates from this information relies on several computational methods, most commonly distance geometry [6] and molecular dynamics [7, 8]. Although these methods have been shown to be robust in the elucidation of solution conformation at low resolution, it is clear that any effort to correlate biological activity with solution conformation and dynamic properties is vitally dependent on the discovery of all possible structures consistent with the NMR data and on some means of filtering quality information (regions which do have a characteristic conformation) from data compromised by conformational heterogeneity.

The issue of completeness in conformational sampling remains an open one. Although distance geometry has been suggested to randomly sample conformational space in much the same fashion as Monte Carlo [9], some investigators have suggested that in the absence of sufficient constraints, it produces fully extended structures that are nearly identical [10]. This suggests that conformational sampling by distance geometry is biased and that care must be exercised in overinterpreting the results of computations for structures or regions of structures that are underdetermined. The complexity of molecular dynamics calculations means that they are time consuming, and consequently it is not possible to run simulations long enough to ensure that all of conformational space is sampled. In addition, weighting of the NMR-derived distance constraints sufficient to ensure convergence to a folded structure may predispose against complete sampling. The problem of becoming trapped in a local minimum has limited the use of optimization methods in generating