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The 3D structure of a cyclosporin analogue in water is nearly identical to the cyclophilin-bound cyclosporin conformation

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Abstract

The conformation of [D-MeSer^3-D-Ser-(*O*-Gly)^8]CS, a water soluble cyclosporin derivative, has been determined in (D_6)DMSO and in water using NMR. In these polar solvents the conformation is identical and very similar to the structure found in the cyclophilin–cyclosporin complex. However, it differs significantly from its conformation in deuterated chloroform. This demonstrates unambiguously that the large structure change is induced primarily by the polar solvent rather than by complex formation with cyclophilin.

Key words: Cyclosporin; Cyclophilin; Affinity; Conformation in water

1. Introduction

Cyclosporin A (CS) [1] (Fig. 1) is a powerful immunosuppressor of T cells [2]. It binds to an endogenous intracellular receptor called cyclophilin A (CYP), and the resulting complex targets the protein phosphatase calcineurin A [3,4]. The structures of CS in single crystals and in apolar solvents have been determined and found to be similar [5–7]. They were described as a rather rigid conformation with three transannular hydrogen-bonds, one *cis* amide linkage and all hydrophobic carbon chains exposed to solvent. The structure of CS bound to its receptor CYP has been revealed by NMR techniques [8–10] and by X-ray analysis of a decameric [11] and of a monomeric [12] CYP–CS complex. The conformation of CS on CYP is significantly different from the structure in single crystals or apolar solvents. It has all peptide bonds in the *trans* form, no intramolecular NH bridged carbonyl and exposes nearly all polar groups to its environment. Another major change is that the MeBmt-1 residue is folded back onto the lower face of the molecule rather than onto the upper face when keeping the orientation of Fig. 1. The OH-group of MeBmt-1 points to the carbonyl of MeLeu-4 and holds the peptide turn of residues 1 to 4 together. This dramatic global rearrangement of the peptide backbone conformation of CS could not be predicted by molecular dynamics calculations [13,14]. It was assumed to be induced by the protein [15,16], but no corroborating evidence was available, and the conformation of uncomplexed CS in water was unknown.

Kinetic and spectroscopic studies [17] with the aid of all-*trans* amide or 9,10-*cis* amide conformations [18,19] of CS showed evidence of a time and solvent dependent inhibition of the peptidyl-prolyl *cis/trans* isomerase activity of CYP by CS and allowed the hypothesis that CYP could bind a conformation of CS already present in water [20], rather than inducing a new conformation. Here we address this problem by describing the NMR structure of [D-MeSer^3-D-Ser(*O*-Gly)^8]CS hydrochloride, a water soluble CS derivative, in (D_6)DMSO and in water.

2. Materials and methods

The cyclosporin derivatives (D-MeSer^3)CS and [D-MeSer^3-D-Ser(*O*-Gly)^8]CS were synthesized starting from CS and (D-Ser^8)CS [21], respectively, using known chemical techniques [1]. (D-Lys^8)CS was obtained from total synthesis [22]. NMR experiments were acquired at 20°C on Bruker AMX400 and AM500 spectrometers. Sample conditions are given in the figure legends. Resonance assignments were obtained from 2QF-COSY [23], TOCSY [24, 25], and NOESY [26] experiments. The data used for structure determination of [D-MeSer^3-D-Ser(*O*-Gly)^8]CS in (D_6)DMSO (4 mM) were obtained from a NOESY spectrum with a mixing time τ_m of 50 ms recorded at 500 MHz. The assigned peaks were integrated using the program EASY [27] and calibrated with the program CALIBA [28] applying a $1/r^6$ relationship between peak volumes and upper distance limits. Supplementary angle constraints were obtained from the program HABAS [29] using *J* coupling constants and

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Abbreviations: CS, cyclosporin A; CYP, cyclophilin A; NOE, nuclear Overhauser effect; RMSD, root mean square deviation.

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the upper distance constraints as input data. Cyclization of the peptide between residues 1 and 11 was imposed by 9 exact distance constraints. 1000 structures were calculated with the program DIANA [28,30] using the standard minimization protocol and one iteration cycle of the REDAC procedure [31]. Stereospecific assignments of β-protons were obtained by analyses of preliminary DIANA structures with the program GLOMSA [28]. To define the set of final structures, the maximum of the pairwise backbone RMSDs was plotted vs. the target function cutoff value and the selection criteria described by Widmer et al. [30] were applied.

3. Results and discussion

NMR results show that a substitution of D-MeSer for Sar in position 3 of CS, as in (D-MeSer^3)CS [1] (Fig. 1), stabilizes a single conformation in (D_6)DMSO and in water compared to multiple conformations of CS in these polar solvents (Fig. 2). On the other side, an amino group in position 8 of CS, as in (D-Lys^8)CS, makes such a derivative water soluble and retains its good affinity for CYP and CS-antibodies [21]. [D-MeSer^3-D-Ser-(*O*-Gly)^8]CS (Fig. 1) contains both modifications, a stabilizing D-MeSer-residue in position 3 and a solubilizing D-Ser-*O*-glycine ester residue in position 8. Its conformation was investigated by NMR spectroscopy in $CDCl_3$, (D_6)DMSO and H_2O. In $CDCl_3$ the well known conformation [5–7] of CS with a 9,10-*cis* amide geometry was found (not shown). However, in the polar solvents used, a different, single conformation predominated (> 95%; Fig. 3). This is in contrast to present knowledge of CS and other CS derivatives which adopt many conformations in polar solvents unless when complexed with a metal cation [18,32] or a protein [8–10]. The conformation of [D-MeSer^3-D-Ser-(*O*-Gly)^8]CS in (D_6)DMSO and in water is the same. This is supported by the finding of only one set of resonances in (D_6)DMSO-water mixtures (Fig. 3) and, moreover, by the coincidence of the short and long range NOEs observed in NOESY spectra measured in both solvents (not shown). Due to this conformational identity in DMSO and water it is sufficient to determine the structure in one solvent only. The conclusions will then be valid for both systems. The complete 3D structure was determined using data collected in (D_6)DMSO. From a NOESY with 50ms mixing time 83 meaningful upper distance constraints were obtained, namely from 38 intraresidual, 30 sequential, and 15 medium and long range NOEs. They were used in distance geometry calculations with the program DIANA. The selected set of final structures consists of 47 conformations (Fig. 4). They have very low residual constraints violations and are well defined (Table 1). The average of the backbone RMSD among all pairs of 47 NMR structures is 0.25 Å. They are nearly identical to the structure of CS complexed to CYP [11,12] (Fig. 4). The pairwise backbone RMSD between the X-ray structure and the individual NMR structures is on average 0.47 Å; the maximum is 0.77 Å, which is similar to the maximum RMSD of 0.72 Å among the NMR structures (Table 1). The hydrogen bond between the β-hydroxyl of MeBmt-1 and the carbonyl of MeLeu-4 observed in the complex

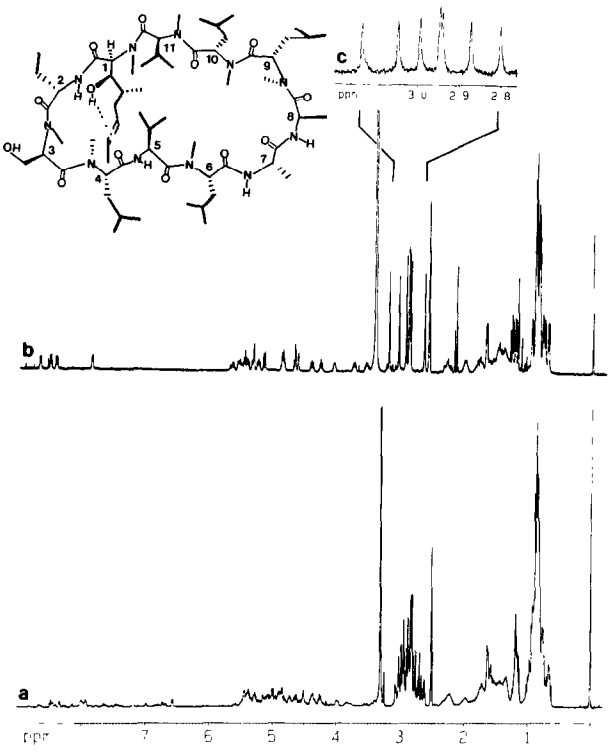

Fig. 2. ^{1}H-NMR spectra (400 MHz) of (a) Cyclosporin A (CS) and (b) (D-MeSer^3)CS in (D_6)DMSO, concentration 4 mM, at 20°C. (c) *N*-Methyl region of an NMR spectrum of a saturated solution of (D-MeSer^3)CS in H_2O, pH 6.9. The stabilizing effect of the D-MeSer residue in position 3 is manifested by observing only one set of resonances (b and c) as compared to multiple sets for CS (a). The chemical structure of (D-MeSer^3)CS is represented in the CYP-bound conformation.

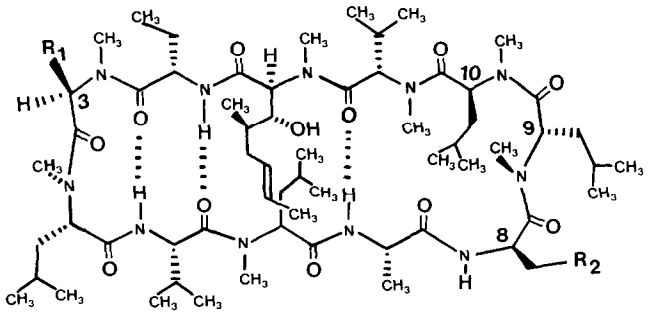

Fig. 1. Chemical structures of Cyclosporin A derivatives (conformation in apolar solvent shown)

Cyclosporin A (CS)	R_1 = H, R_2 = H	
(D-MeSer^3)CS	R_1 = CH_2OH, R_2 = H [1]	[1]
(D-Lys^8)CS·HCl	R_1 = H, R_2 = $(CH_2)_3NH_2$·HCl	[22]
[D-MeSer^3-D-Ser(*O*-Gly)^8] CS·HCl	R_1 = CH_2OH, R_2 = O-CO-CH_2NH_2·HCl	[1]

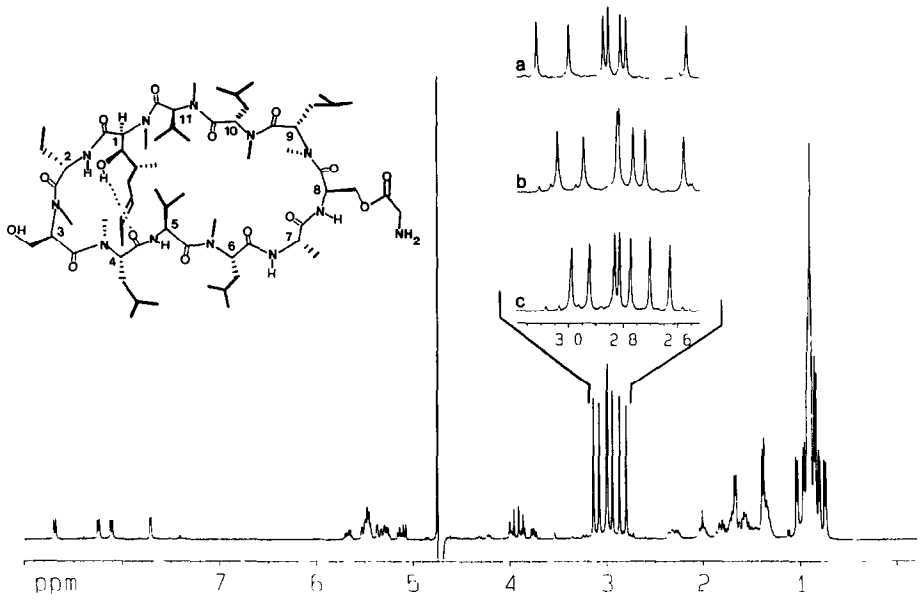

Fig. 3. ^{1}H-NMR spectrum of [D-MeSer^3-D-Ser(*O*-Gly)^8]CS.HCl in water at 400 MHz and 20°C, concentration 4 mM, pH 5.6 (addition of NaOH to give pH 7.0 gave an identical spectrum). In the insert (a–c) the *N*-methyl regions of NMR spectra measured in solvent mixtures [34] are shown; (a) (D_6)DMSO, (b) (D_6)DMSO: H_2O (4:1), (c) (D_6)DMSO: H_2O (1:4). The chemical structure of [D-MeSer^3-D-Ser(*O*-Gly)^8]CS is represented in the CYP-bound conformation.

with CYP [11,12] is also found in 26 of the 47 uncomplexed solution structures. The CYP-bound CS conformation fits into the envelope of the NMR structures at all residues except MeVal-11 (Fig. 4). The displacement between the C^α position in the average NMR structure and the corresponding C^α atom in the X-ray structure is 0.83 Å for MeVal-11 and < 0.6 Å for all other residues. This structure determination of [D-MeSer^3-D-Ser-(*O*-Gly)^8]CS provides, for the first time, evidence that a free CS analogue adopts the CYP-bound CS conformation in (D_6)DMSO and in water. The concept of a 'receptor-induced conformation' [15,16] applies only to MeVal-11 which is 'sucked' into the binding pocket of CYP.

The equilibrium affinity constants for (D-MeSer^3)CS and [D-MeSer^3-D-Ser(*O*-Gly)^8]CS to CYP initially dissolved in DMSO have been found to be about 10 times higher than that of CS (Van Regenmortel et al., to be published). The kinetics of CYP binding to [D-MeSer^3-D-Ser(*O*-Gly)^8]CS was monitored by measuring 1D NMR and found to be 90% complete within three minutes (not shown). This is in contrast to CS, which requires 30–45 min for complex formation with CYP [17,33] and supports the hypothesis that CS has to undergo structural changes before complex formation with CYP in a rate limiting step.

Our results confirm that the cyclophilin bound conformation is a good model for discussing structure activity relationships of cyclosporins and show, for the first time, that through a minor structure change in position 3, it is possible to obtain the cyclophilin-bound cyclosporin conformation *free* in water.

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Table 1
Analysis of the 47 NMR structures of [D-MeSer^3-D-Ser(*O*-Gly)^8]CS in (D_6)DMSO and comparison with the X-ray structure of CS in the CYP complex [12]

Parameter	Average value (min, max)
DIANA target function (Å^2)	0.29 (0.19, 0.37)
Residual NOE violations (Å)	
Sum	0.55 (0.3, 0.9)
Maximum	0.14 (0.07, 0.29)
Residual Van der Waals violations (Å)	
Sum	1.3 (1.0, 1.6)
Maximum	0.16 (0.14, 0.22)
Pairwise RMSDs (Å): NMR structures	
Backbone atoms (N, C^α, C')	0.25 (0.04, 0.72)
Heavy atoms*	0.57 (0.21, 1.18)
Pairwise RMSDs (Å): X-ray vs. NMR structures	
Backbone atoms (N, C^α, C')	0.47 (0.35, 0.77)
Heavy atoms*,+	0.75 (0.59, 1.10)

*Only atoms up to the γ-positions of the side chains are included, so that variants of the χ^2 angles and beyond are not considered.
+In residues 3 and 8 only atoms which have a corresponding atom in CS are considered.

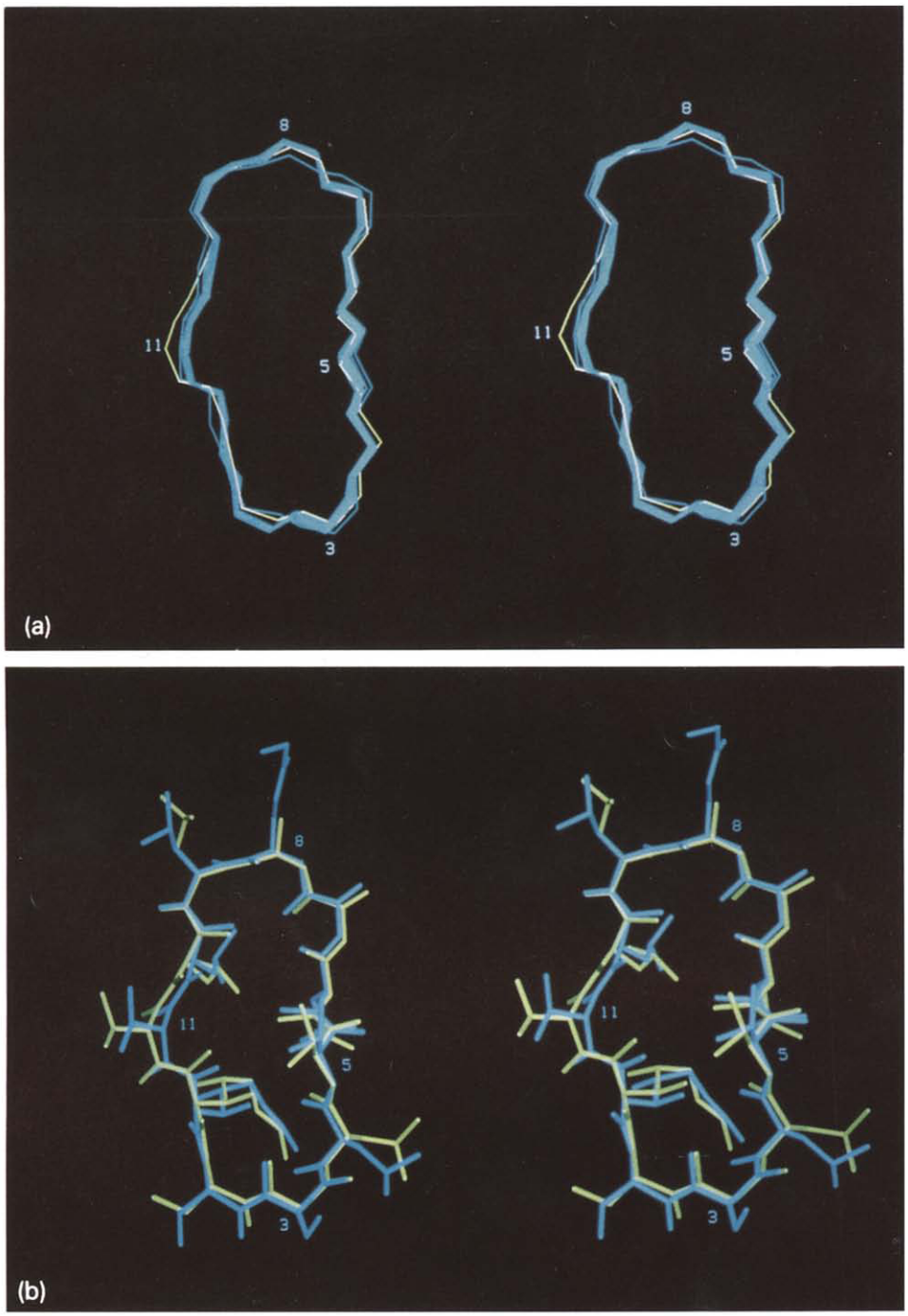

Fig. 4. Stereoview of the NMR structures of uncomplexed [D-MeSer^3-D-Ser(*O*-Gly)^8]CS in (D_6)DMSO solution (cyan) superimposed with the X-ray structure of cyclophilin-bound CS [12] (yellow). (a) Representation with backbone atoms C′,C^α, and N; 47 NMR structures are shown. (b) Heavy atom representation; the NMR structure with lowest backbone RMSD (0.35 Å) to the X-ray structure is shown; the target function value is 0.30 Å^2.

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