NMR Studies of the Na^+, Mg^{2+} and Ca^{2+} Complexes of Cyclosporin A

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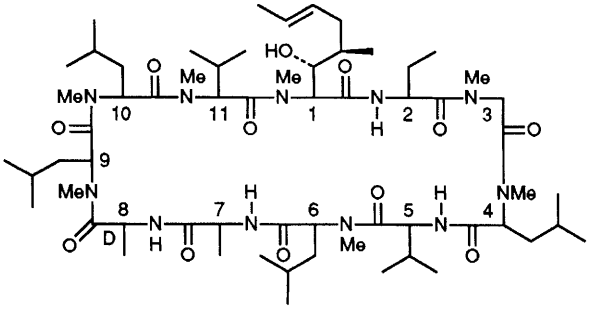
The immunosuppressant cyclosporin A has been shown by NMR spectroscopy to form complexes with Na^+, Ca^{2+} and Mg^{2+} in acetonitrile; Ca^{2+} and Mg^{2+} form 1 : 1 complexes and the conformation of cyclosporin A changes dramatically upon binding of the metal ions.

The important immunosuppressive cyclic undecapeptide cyclosporin A (CsA) has been the focus of great interest for several years.[1] In order to try to relate the biological activity of CsA to its structure, NMR spectroscopy has been used to determine its conformation in low polarity solvents such as $CDCl_3$ and C_6D_6[2,3] and the results have been compared with the X-ray structure analysis.[3] Recent NMR studies have reported the conformation of CsA bound to its presumed receptor protein, cyclophilin, in aqueous solution.[4] There is good overall agreement between these independent studies of bound CsA, though there are significant conformational differences which may result from the different datasets or differences in the computational methods. The backbone conformation of bound CsA was found to be substantially different from that found in CsA crystals and in non-polar solutions of free CsA. For example, all the peptide bonds have the *trans*-configuration in CsA bound to cyclophilin whereas there is one *cis*-peptide bond between residues MeLeu9 and MeLeu10 in the unbound form. Recent measurements by circular dichroism have revealed that CsA forms complexes with the ions Na^+, Mg^{2+} and Ca^{2+},[5] and the NMR studies presented here show that these complexes have conformations which differ from each other and from that of free CsA. Organic solvents have been used for all these studies except those of cyclophilin–CsA since CsA has an extremely low solubility in water and its behaviour in a 'lipophilic' solvent such as acetonitrile may be more physiologically relevant.[3]

CsA was dissolved in trideuterioacetonitrile at a concentration of 50 mg ml^{-1} and 0.6 ml samples in 5 mm tubes were used for NMR measurements with a Varian VXR500 spectrometer. Various concentrations of metal ions were produced by addition of analytical grade crystalline perchlorates to CsA samples.

Addition of approximately 0.33 mol. equiv. of magnesium perchlorate to a solution of CsA produced a series of extra ^1H resonances superimposed on the spectrum of unbound CsA, showing that exchange between the Mg^{2+} complex and the free form is slow on the NMR time scale. The additional peaks increased in intensity (Fig. 1) as further $Mg(ClO_4)_2$ was added and the spectrum of the free form had essentially disappeared at a 1 : 1 mol ratio. Further addition of $Mg(ClO_4)_2$ caused no further change in the spectrum, other than slight shifts which may be attributed to the ionic strength, indicating that a 1 : 1 complex is formed. The addition of calcium perchlorate to a solution of CsA gave similar results to those for the Mg^{2+} complex, *i.e.* a 1 : 1 complex formed upon addition of an equimolar quantity of $Ca(ClO_4)_2$. On the other hand, 10 equiv. of sodium perchlorate were needed before a single species was present in solution. The spectrum of the sodium complex did not change significantly with further additions of $NaClO_4$ and, although only one form of complex is observed, the stoichiometry remains uncertain. Finally, the spectrum of CsA in the presence of 1 equiv. of $Mg(ClO_4)_2$ and of $Ca(ClO_4)_2$ exhibited resonances for both the Mg^{2+} and Ca^{2+} complexes of approximately equal intensities. Thus, it is clear all three ions form complexes with CsA and the order of binding constants is $Na^+ \ll Mg^{2+} \approx Ca^{2+}$.

Further experiments used samples with the minimum salt concentrations necessary to complex all the CsA, *i.e.* 1 : 1 $Mg(ClO_4)_2$ and $Ca(ClO_4)_2$ and 10 : 1 $NaClO_4$. Standard procedures for sequential assignment[6] were used to analyse the ^1H spectra obtained with TOCSY and ROESY pulse sequences.[7] A lack of significant scalar couplings between the *N*-methyl and α-protons made it necessary to refer to the ^{13}C spectra to obtain these ^1H assignments. Heteronuclear multiple-quantum coherence (HMQC)[8] was used for cross-assignment and long range ^1H–^{13}C couplings were detected by using an additional pulse to suppress correlations between directly bonded nuclei (HMBC).[9] The $^3J_{C\text{-}H}$ intra-residue couplings



Cyclosporin A

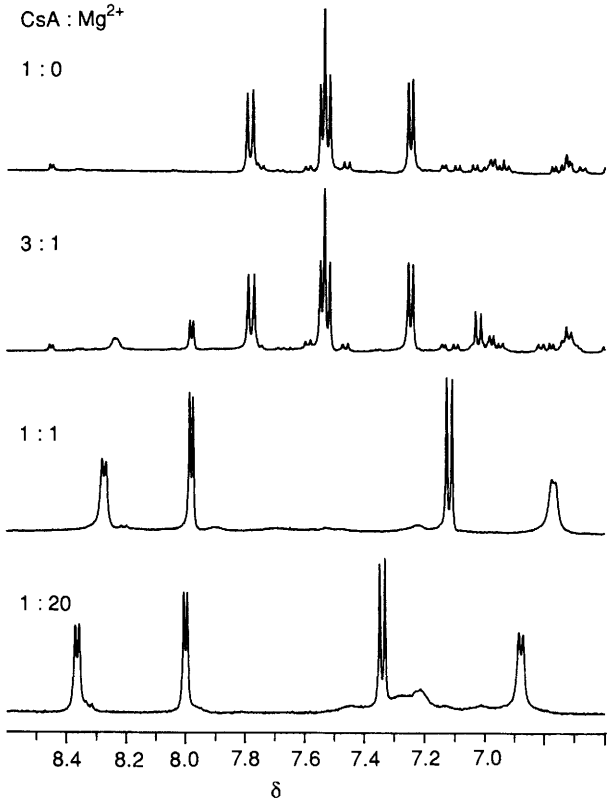


Fig. 1 The NH region of the ^1H NMR spectrum of CsA in CD_3CN at 293 K showing the effect of adding increasing quantities of $Mg(ClO_4)_2$. Several lines of low intensity arise from minor conformations of unbound CsA (*cf.* ref. 3).

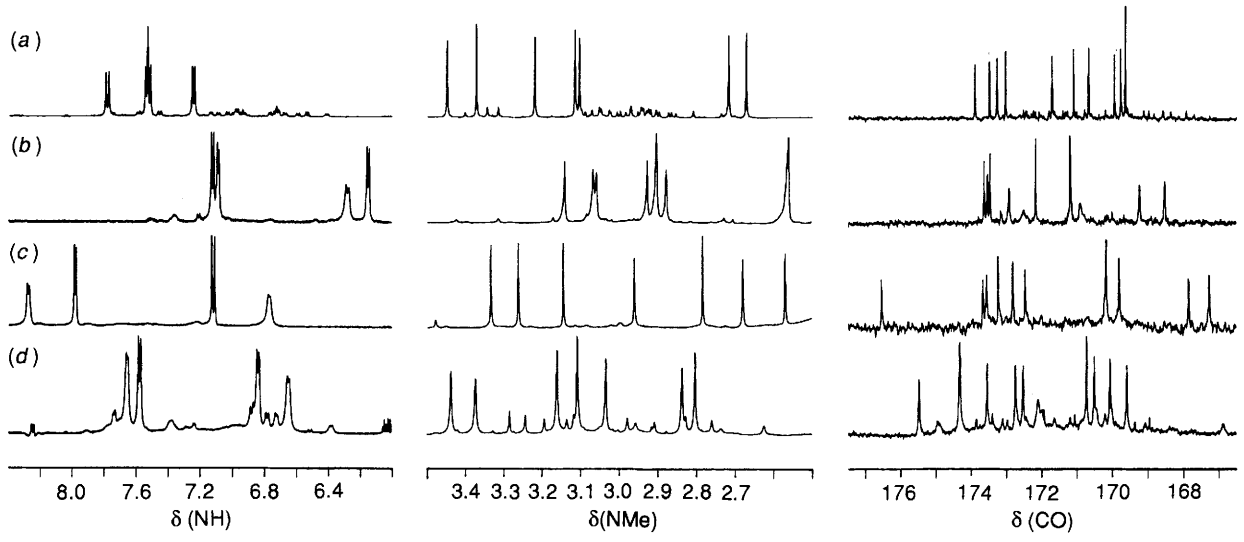


Fig. 2 Comparison of the NH and NMe regions of the ^{1}H NMR spectra and the carbonyl region of the ^{13}C NMR spectra of uncomplexed and complexed CsA at 50 mg ml^{-1} in CD_3CN at 293 K: (*a*) free CsA, (*b*) 1:10 CsA–Na^+, (*c*) 1:1 CsA–Mg^{2+}, (*d*) 1:1 CsA–Ca^{2+}

Table 1 ^{1}H chemical shifts (δ) of NH, NMe and αCH protons, ^{13}C chemical shifts (δ) of carbonyls, and $^3J_{NH\text{-}\alpha CH}$ (in Hz) for uncomplexed and complexed CsA in CD_3CN at 293 K

		Free CsA	CsA–Na^+	CsA–Mg^{2+}	CsA–Ca^{2+}
MeBmt 1	NMe	3.45	3.08	2.96	3.35
	αCH	5.38	5.45	4.25	4.20
	CO	169.85	172.53	167.35	172.12
Abu 2	NH	7.79	7.10	6.77	7.67
	αCH	5.03	4.66	4.77	4.71
	CO	173.35	173.66	173.75	174.34
	$^3J_{NH\text{-}\alpha CH}$	9.6	6.0	7.6	6.3
Sar 3	NMe	3.37	3.14	3.33	3.18
	αCH	4.72	4.83	4.71	5.23
	CO	171.74	168.55	170.23	172.77
MeLeu 4	NMe	3.10	2.94	2.68	3.03
	αCH	5.32	5.12	4.39	4.52
	CO	169.73	169.26	173.65	172.55
Val 5	NH	7.53	6.30	8.27	6.69
	αCH	4.66	4.71	3.76	4.26
	CO	173.98	173.56	176.62	175.50
	$^3J_{NH\text{-}\alpha CH}$	8.4	9.2	6.8	7.3
MeLeu 6	NMe	3.22	3.08	3.26	3.43
	αCH	5.07	5.27	4.17	3.80
	CO	171.19	171.23	172.90	170.74
Ala 7	NH	7.56	6.16	7.98	7.57
	αCH	4.36	4.31	4.40	4.21
	CO	170.76	172.20	169.88	169.60
	$^3J_{NH\text{-}\alpha CH}$	6.9	5.1	5.8	7.6
D-Ala 8	NH	7.26	7.14	7.12	6.81
	αCH	4.80	4.66	5.11	4.91
	CO	173.55	173.49	173.31	173.56
	$^3J_{NH\text{-}\alpha CH}$	7.6	7.3	9.0	7.6
MeLeu 9	NMe	3.12	2.88	3.15	3.11
	αCH	5.70	5.48	5.71	5.65
	CO	170.04	172.23	170.27	170.52
MeLeu 10	NMe	2.67	2.93	2.79	2.81
	αCH	5.09	5.51	5.14	5.19
	CO	169.73	172.95	167.95	170.08
MeVal 11	NMe	2.72	2.93	2.57	2.84
	αCH	5.19	5.12	5.10	5.20
	CO	173.10	170.92	172.56	174.34

between *N*-methyl protons and α-carbon nuclei proved necessary to assign all the *N*-methyl groups. The carbonyl groups showed heteronuclear couplings to the NH or *N*-methyl protons of the $n + 1$ residue as well as weaker intra-residue cross peaks to the α-proton and, where present, NH.

It is apparent from Fig. 2 and Table 1 that there are significant variations in the chemical shifts of the backbone resonances of the different metal complexes and of free CsA in CD_3CN. The pattern of $^3J_{NH\text{-}\alpha H}$ coupling constants shown in Table 1 also varies, showing that there are significant differences in conformation between the different forms of complexed and uncomplexed CsA. This supports the conclusions based on circular dichroism measurements.[5] There are some differences in chemical shift between solutions of CsA in CD_3CN and those in $CDCl_3$ and C_6D_6[2,3] and tetrahydrofuran (THF)[10] which were reported previously, but a similar pattern of NOEs is found, in each case. This suggests that the conformation of free CsA is similar in each of these solvents, which is in agreement with the results from circular dichroism.[11] The addition of LiCl to CsA dissolved in THF has also been shown to result in the formation of a complex.[10]

The patterns of NOEs observed in rotating frame (ROESY) experiments are quite different for the free and complexed forms of CsA. This is shown most clearly by the αH_n–αH_{n+1} NOEs which are diagnostic of the presence of *cis*-peptide bonds. Free CsA has one *cis*-peptide bond linking residues 9 and 10, and the magnesium and calcium complexes have a *cis*-peptide bond in the same position. However, the magnesium complex has an additional *cis*-peptide bond linking residues 3 and 4, and all the bonds in the sodium complex are *trans*.

These observations are of critical importance in understanding the mode of action of CsA since it is not certain whether the active form is the free peptide or a metal complex. The maximum concentration of CsA likely to be found therapeutically is 8 μmol l^{-1} (based on a dosage of 10 mg per kg bodyweight)[12] whereas the concentrations of free Ca^{2+} and Mg^{2+} in plasma are of the order of 1 mmol l^{-1}.[13] It is quite possible, therefore, that either the CsA–Mg^{2+} or the CsA–Ca^{2+} complex is the active form, so that their conformations and biological activity should be a matter of considerable interest.

In summary, we have determined that cyclosporin A forms

strong 1 : 1 complexes with Mg^{2+} and Ca^{2+} as well as a weaker complex with Na^{+}. All these complexes have distinct conformations, which are being elucidated at present and will be reported in detail elsewhere.

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