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Conformational and ion-binding properties of cyclolinopeptide A isolated from linseed

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Abstract. The conformation of the cyclic nonapeptide from linseed, cyclolinopeptide A in methanol and in acetonitrile has been elucidated by one- and two-dimensional nuclear magnetic resonance. The molecule is folded in a *ß*-turn conformation. Cyclolinopeptide A interacts and weakly complexes with Tb^{3+} (a Ca^{2+} mimic ion) with the metal ion positioned proximally to the Phe residue, but with no substantial structural alteration upon metal binding. Cyclolinopeptide A is also seen to aid the translocation of Pr^{3+} (another Ca^{2+} mimic) across unilamellar liposomes. However, cyclolinopeptide A does not phase transfer or act as an ionophore of calcium ion myself. Experiments using lanthanide ions thus do not necessarily indicate any ionophoretic ability of the complexone towards calcium ions.

Keywords. *ß*-Turn and calcium ion binding; one- and two-dimensional nuclear magnetic resonance of peptides; ionophoretic assay; lanthanide probes; fluorescence energy transfer.

Introduction

Cyclolinopeptide A (CLPA) is a homodetic cyclic nonapeptide from linseed (Kaufmann and Tobschirbel, 1959) and has the structure I. Its partial homology with the ionophore antamanide (structure II) has evoked interest in the conformation, ionophoretic properties and the biological role of CLPA. Early work on its spectral and conformational analysis (Naider *et al*., 1971; Brewster and Bovey, 1971; Tonelli, 1971) suggested a flexible conformation for CLPA in solution. Siemion *et al*. (1977) had suggested that CLPA might adopt a *ß*-turn conformation. They also found the peptide to complex weakly with K^+ ions, with little conformational change upon ion binding. Our own studies around the same time (Balasubramanian *et al*., 1976) showed that CLPA adopts 3 broad classes of conformations (termed A, B and C) in solution, binds Ca^{2+} ions weakly, and changes from the B conformation into the A form upon binding this ion in methanol.

We present here results of our further studies on the conformation of CLPA and its ion binding propensities. This is motivated particularly by the growing evidence for the suggestion that the *ß*-turn region of a peptide chain offers itself as a favourable ligand for the binding of Ca^{2+} and like ions (Vogt *et al*., 1979; Ananthanarayanan *et al*., 1985), and it would be worthwhile testing this suggestion with CLPA. In this context, we have identified the *ß*-turn region in CLPA using the method of one- and

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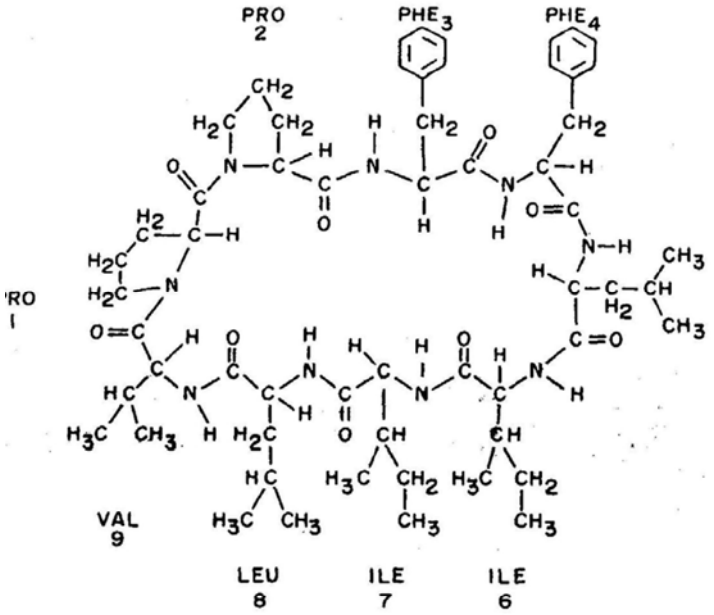
Abbreviations used: CLPA, Cyclolinopeptide A; NMR, nuclear magnetic resonance; ULV, unilamellar vesicles; COSY, correlation spectroscopy; NOE, nuclear Overhauser effect; DMPC, dimyristoyl phosphocholine.



I (CLPA)

Phe — Phe — Pro — Pro — Phe
Ala — Pro — Pro — Val — Phe

II

(ANTAMANIDE)

two-dimensional nuclear magnetic resonance (1D and 2D NMR), located the site of metal ion interaction in the peptide, using the fluorophore Tb^{3+} which is an excellent Ca^{2+} mimic (Brittain *et al*., 1976; Horrocks *et al.,* 1984) and monitored the ion-translocating ability of CLPA across unilamellar lipid vesicles (ULV) using Pr^{3+} ion, another Ca^{2+} mimic (Sankaram, 1983; Sankaram *et al*., 1985). However Ca^{2+} does not appear to bind to CLPA nor does it get translocated across ULVs by the peptide. The ions Tb^{3+} and Pr^{3+} also bind only weakly to CLPA, essentially as outer sphere complexes. It thus appears that while these ions may mimic Ca^{2+}, they display special properties such as outer sphere complexation (which Ca^{2+} cannot), and hence the use of these ions for assaying the Ca^{2+} ionophoretic activity is not fool proof.

Materials and methods

CLPA was isolated from linseed cake by Soxhlet extraction from acetone and recrystallized from acetone-ether, m.p. 243°C. $TbCl_3$ was a product of E. Merck, Germany and CD_3OD (99·5%) was from Aldrich Chemical Company, USA. All other chemicals used were of purest grade available. Uncorrected fluorescence emission spectra were

recorded on a 650–10S Hitachi spectrofluorimeter, with care taken to avoid the inner filter effect. [^{1}H]-NMR spectra were obtained using a Broker 500 MHz FT NMR spectrometer. Two dimensional J-resolved correlation spectroscopy (COSY) was done at 24°C following the standard pulse sequence discussed in detail elsewhere (Nagayama *et al*., 1977; Benn and Gunther, 1983; Redfield and Kunz, 1979). The spectral width was 8064·516 MHz. 32 free induction decays were accumulated for each value of t_1 with 400 experiments or t_1 values in the time domain. For the nuclear Overhauser effect (NOE) experiments, a given peak of interest was selectively saturated by gated irradiation at that frequency, by using just enough decoupler power to almost saturate the resonance in 0·1 s. The NOE data were collected directly by interleaving, as NOE difference spectra, by using difference Fourier techniques (Tropp and Redfield, 1981). Five pulses irradiated on the resonance line and the same number of pulses irradiated off resonance were interleaved in an alternating cycle, with memory negation, to generate the difference free induction decay which was Fourier transformed to generate the difference spectrum in the frequency domain.

Results and discussion

Fluorescence energy transfer between CLPA and Tb^{3+}

$TbCl_3$ displays an inherent fluorescence emission spectrum with characteristic maxima at 485 nm and 540 nm, when excited at 290 nm but only when present at sufficiently large concentrations. However, at a concentration below 1 mM in methanol or in acetonitrile, no fluorescence signals were observed at the above region indicating thereby that the enhancement of Tb^{3+} fluorescence at this concentration range could be conveniently studied if it binds to the peptide. The fluorescence spectrum of CLPA itself in MeOH showed a characteristic emission band at 284 nm when excited at 257 nm, typical of the phe moiety in the peptide. The matching of the emission band of CLPA and the excitation band of Tb^{3+} makes it possible to monitor any energy transfer between the peptide and the ion and to investigate the binding process if that occurs. Figure 1 shows the enhancement of the 540 nm emission band of Tb^{3+} and the concomitant quenching of the 284 nm band of the peptide that occur as $TbCl_3$ is added to a solution of CLPA in methanol. Since the fluorescence intensity of the phe moieties in CLPA is orders of magnitude more than that of the CLPA: Tb^{3+} system, any energy transfer would be expected to only negligibly affect the phe emission band at 284 nm. However, the phe-sensitized emission in Tb^{3+} is seen to be quite appreciable at 540 nm, which results in a small but significant drop in the peptide emission at 284 nm. It appears that the maximal fluorescence change could be observed at a mol ratio of 5:1 CLPA: $TbCl_3$. Also, identical results were obtained with acetonitrile as the solvent. It has been reported earlier that the conformation of CLPA is the same in acetonitrile and in alcohols (Balasubramanian *et al*., 1976).

It is noteworthy that the fluorescence of Tb^{3+} is substantially enhanced in the presence of CLPA. This would happen if energy transfer occurs from the peptide to the ion, or if the ion were to be bound to the peptide in close proximity. The fact that neither $TbCl_3$ nor the CLPA:Tb^{3+} system display any significant absorption

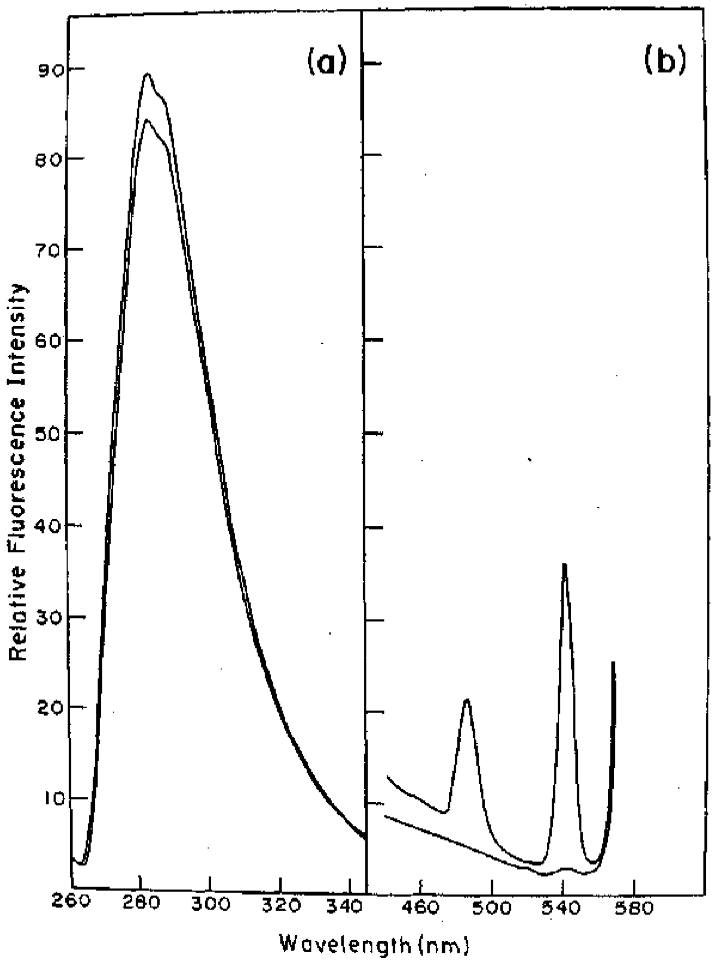


Figure 1. (a), Fluorescence spectrum of CLPA, upon excitation at 257 nm. The top curve is for the free peptide in methanol (1 mM concentration) and the lower curve is for the system CLPA: $TbCl_3$ (5:1 mol ratio). (b), The emission spectrum of $TbCl_3$ (0·2 mM) in methanol, in the presence and absence of CLPA. Excitation wavelength 290 nm. The lower curve shows the spectrum of $TbCl_3$ alone while the upper curve is for the system CLPA:$TbCl_3$ (5:1 mol ratio). The peptide myself does not fluoresce in this region.

intensity in the 285–290 nm region (the emission region of the peptide) makes any dipole-dipole or Forster type energy transfer unlikely; instead, a close proximity between the Tb^{3+} ion and the phe residues of CLPA, *i.e.*, binding of the ion, is indicated.

Though substantial spectral changes occur when $TbCl_3$ is added to CLPA, measurements of the stability constant of the complexation and its stoichiometry either by fluorimetry or electrical conductivity showed very weak complexation and no definite stoichiometry between $TbCl_3$ and CLPA. This behaviour of CLPA is akin to that of antamanide and lanthanide ions (Wieland *et al.*, 1972), and indicates that the ion complexation to CLPA is unspecific and an outer sphere complexation by the metal ion.

Conformational features by NMR spectroscopy

The fluorescence results and our earlier circular-dichroism results (Balasubramanian *et al*., 1976) suggest that CLPA displays detectable spectral changes in the presence of certain ions, but it is not clear whether these changes reflect conformational alterations in the molecule or are due to a medium effect. In order to investigate these possibilities further, we have used 1- and 2-D NMR spectral methods. Figure 2 shows the J-resolved correlation spectrum (2D-COSY) of CLPA in the solvent

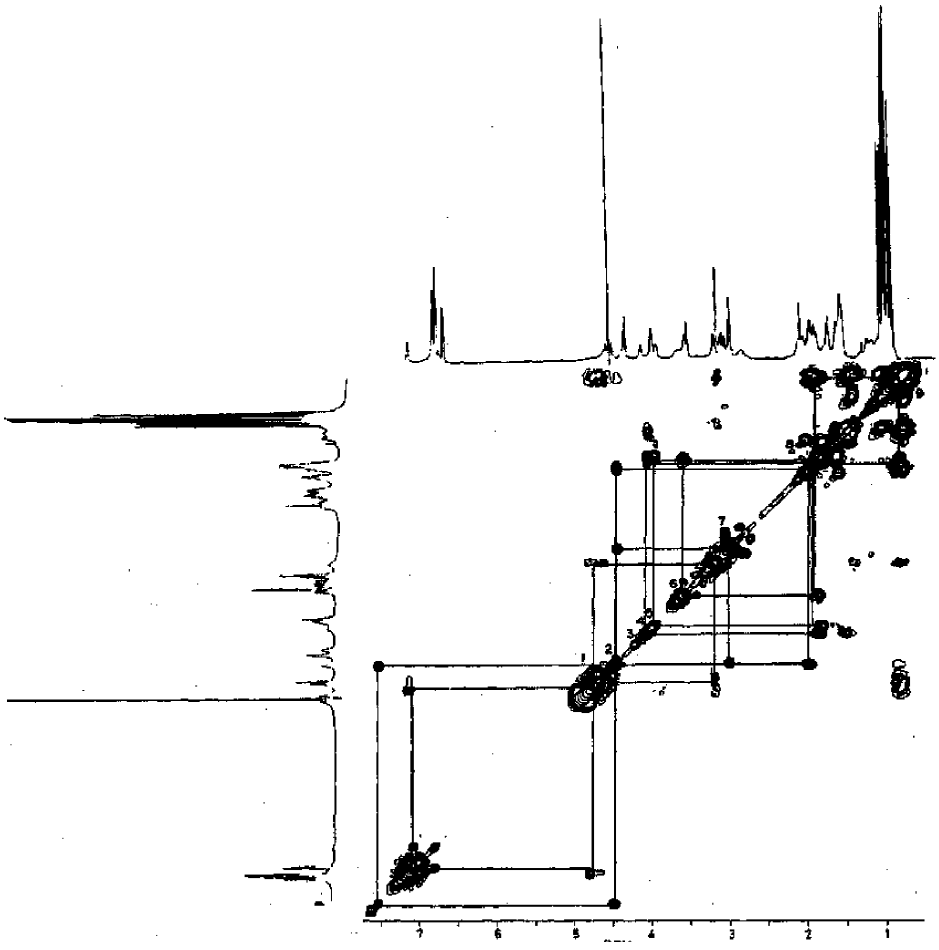


Figure 2. 500 MHz 2-D J-resolved correlation spectroscopy, 2-D COSY plot of CLPA (1 mM) in CD_3OD. The connectivities of the coupled protons are indicated by the square grids and the protons assigned are designated by numbers as under: (1), Ile- α-CH; (2), phe- α-CH, val- α-CH; (3), leu- α-CH; (4), pro- α-CH; (5), Ile- α-CH; (6), pro- δ-CH; (7), phe- *β*-CH; (8), val- *β*-CH; Ile- *β*-CH; and (9), val- γ, δ-CH, Ile- γ, δ-CH. The assignment of the relevant amide protons (phe-NH and Ile-NH) in the 7 ppm region follows from the connectivity diagrams. The spectrum was symmetrised to remove solvent peak and the strong methyl peaks below 1 ppm. The size of the spectrum in the frequency domain had 2048 data points (ω_2) and 1024 data points (ω_1), and the value was 7·876 Hz per point.

CD_3OD. This solvent was used since all exchangeable protons in CLPA would be deuterated here, thereby simplifying the spectrum. In the assignment of the peaks, we have been aided by the earlier work (Brewster and Bovey, 1971; Siemion *et al*., 1977). The δ-CH_2 protons of pro residues are the easiest to assign since they stand apart around 3·5 ppm, and are J-coupled to the pro-γ protons that occur alongwith the pro -*β* protons which are in turn coupled to the pro-α CH. The later will of course not be coupled at any NH protons. Thus, one of the cross peaks in the COSY spectrum at 4·2 ppm is assignable to pro-α CH. The assignment of the signal at 7·55 ppm to a phe-NH proton is also easy since it is correlated to the phe α-CH proton, which should be the most downfield shifted of the α-CH manifold in the 4·2–4·8 ppm range. This assignment of the phe-NH and the phe-α CH is consistent with the earlier suggestion that one of these aromatic NH(phe-3) is intramolecularly H-bonded (Siemion *et al*., 1977), and would not be solvent-exchanged. This was further confirmed by a double irradiation experiment, where selective saturation of the 7·5 ppm signal resulted in a simplification of the α-CH manifold in the 4·5–4·8 ppm region. Further identification of the phe protons was also achieved by locating the phe -*β* CH_2 through the correlation diagram in figure 2. The second signal below 7·0 ppm region was identifiable as due to Ile-NH, following Siemion *et al*. (1977). All the other assignments, as shown in figure 2, are straight forward and the ambiguities could be resolved by doing 1-D NOE experiments.

Such a 1 -D NOE, coupled with double irradiation experiments as well, has suggested that a Val- α-CH occurs close to the phe- α-CH, and is coupled to the val-*β*-CH_2, and the correlation of the latter to the γ- and these to the ω-protons. The leu-*β*-CH that gives rise to a weak cross peak could be assigned as per Brewster and Bovey (1971). The Ile- α-CH occurs just below the pro-α-CH, and could be assigned again following the connectivity diagrams. The assignments are shown in figure 2.

It was earlier surmised (Siemion *et al*., 1977) that Ile-7-NH is also intramolecularly H-bonded, but its assignment proved difficult due to overlap by the aromatic protons in the 7·1 ppm region (see above). We have been able to clarify and confirm this by using the Tb^{3+} induced shifts that occur in the NMR spectrum of CLPA in presence of this ion. Figure 3 shows the spectrum of the system 5:1 CLPA: $TbCl_3$. The aromatic region is better resolved here than in free CLPA and there are minor changes upon ion binding, very likely due to the proximal interaction between the ion and the phe ring. A sharp multiplet appears between the solvent peak and the α-CH signals around 4·5 ppm. Now when the signal at 7·7 ppm is irradiated, the multiplicity at 4·8 ppm is reduced into a singlet. Based on the assignment of the 4·8 ppm signal to the Ile- α -CH (see above), the 7·7 ppm signal in the CLPA: $TbCl_3$ system is assigned to the Ile-NH signal, which has been downfield shifted by the lanthanide ion. Other than this shift, no other changes are seen in the NMR spectrum of CLPA upon the addition of this ion, reflecting the absence ofany significanct change in the peptide conformation upon Tb^{3+} binding. Such would be the case if the ion were to form an outer sphere complex with the peptide. Incidentally, no spectral changes were observed between free CLPA and when Ca^{2+} was added to a solution of the peptide, suggesting that Ca^{2+} ion is not bound any better than Tb^{3+} to CLPA.

We also monitored the isomerization status of the pro residue in CLPA in the free form and in the presence of $TbCl_3$. We observed a proton spin-spin coupling constant value of $J_{\alpha\ CH,\ \beta\text{-}CH_2} = 8$ Hz in both instances for the pro signals. This argues

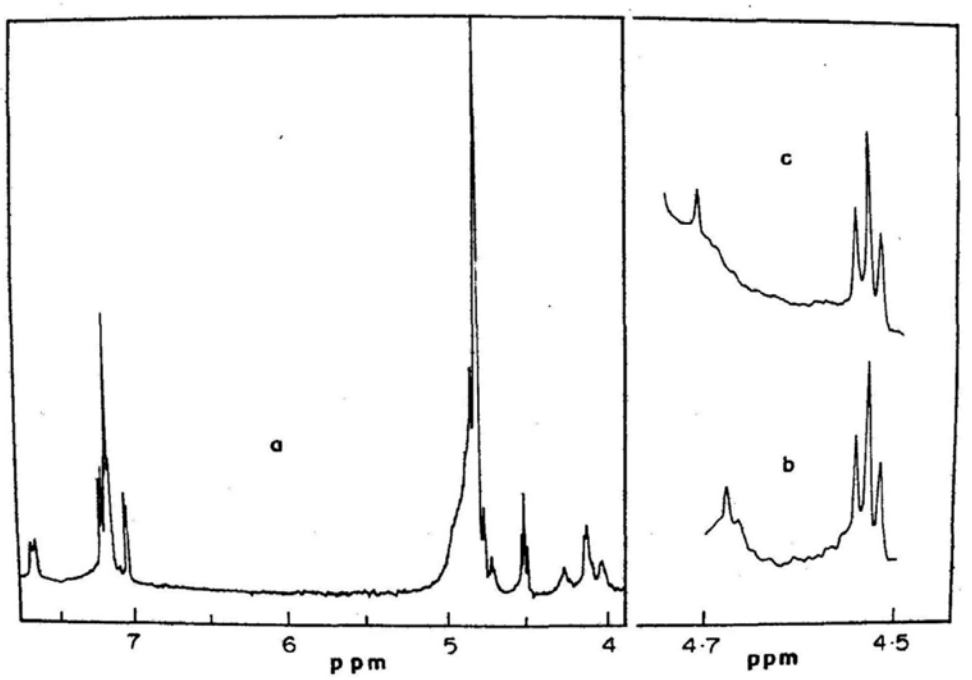


Figure 3. 1D-NMR spectrum of CLPA (1 mM) in CD_3OD in the presence of $TbCl_3$ (0·3 mM). (a), Downfield region of the system; (b), the α-CH region; (c), is the α-CH region with double irradiation at 7·18 ppm.

for at least one of the residues to be in the *cis* (Patel, 1973; Anteunis *et al*., 1975), is in accordance with the [^{13}C]-NMR data (Siemion *et al*., 1977), and indicates that this isomer status is unchanged upon Tb^{3+} binding. The presence of a cis pro residue and the hydrogen bonding through a phe-NH would argue for a *β*-turn conformation in this region of CLPA.

Further support for this conformational assignment comes from the 2D-COSY spectra of the peptide in the solvent CD_3CN where its conformation is known to be the same as in alcohols. Figure 4a shows that it is possible to resolve and assign all the NH protons of the molecule in this non-exchanging solvent. Such assignment, following the connectivity patterns as in the CD_3OD case, uses the pro-δ-CH which stands apart between the α-CH and *β*-CH regions and which can be connected successively through the neighbouring protons of the residue. The val and phe-α-CH signals around 4·2 ppm and can be individually located since the phe- *β*-CH_2 that connects the latter is the most downfield shifted in its class and has no other connectivity. Therefore, the corresponding NH protons are also easily assigned. Following Brewster and Bovey (1971), one of the Ile-NH should be more downfield shifted, and, as the two Ile- α-CH absorb together, they can be assigned as also their NH protons. The leu protons are then assigned essentially by a process of elimination. All the assignments are indicated in figure 4a for the free peptide.

Upon the addition of $TbCl_3$, the phe- α-CH is downfield shifted compared to the val- α-CH (see figure 4b) and a reduction in the aromatic manifold occurs. While the changes in the phe-NH signal are a little difficult to monitor, the downfield shift of

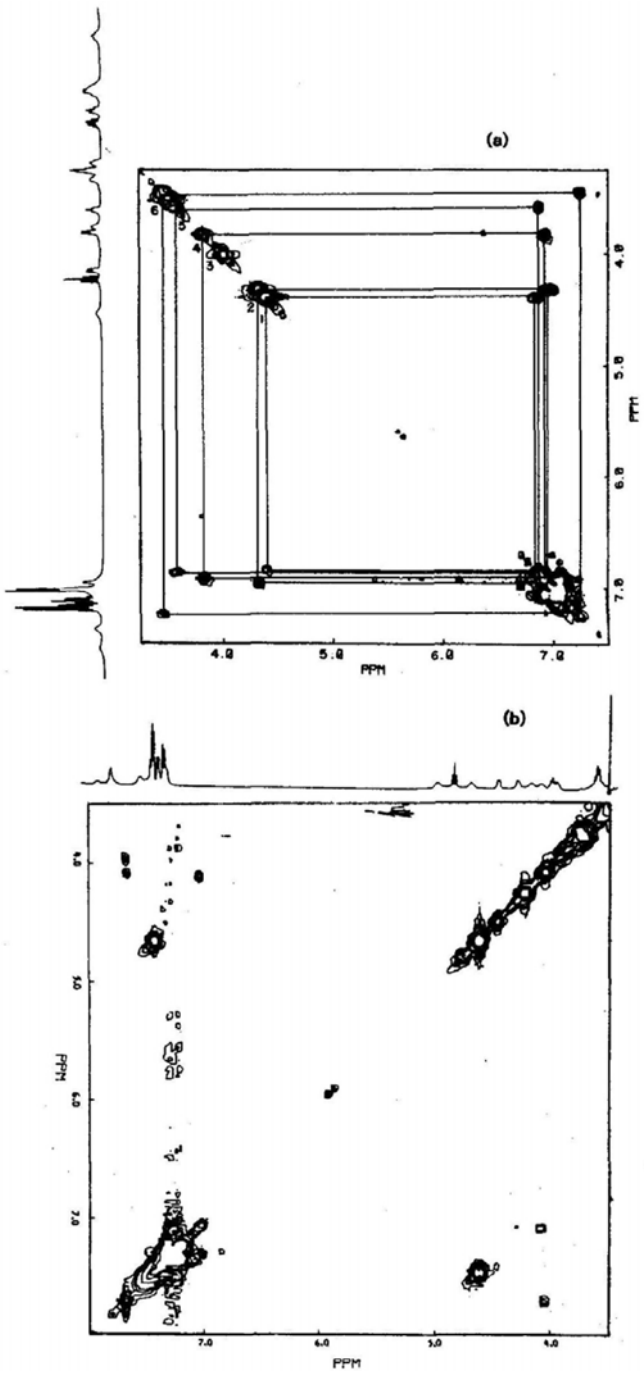


Figure 4. (a), 2D-COSY plot of CLPA (1 mM) in CD_3CN. The conditions and the display are the same as in figure 2 and the assignments are as under: (1), val- α-CH; (2), phe- α-CH; (3), pro- α-CH; (4), leu- α-CH; (5), Ile- α-CH and (6), Ile- α-CH. The corresponding NH proton assignments in the 7 ppm region follow from the connectivity diagrams, (b), 2D-COSY plot of CLPA (1 mM) in CD_3CN in the presence of $TbCl_3$ (0·2 mM). Notice the Tb^{3+}-induced downfield shifting of the phe- α-CH and reduction in the aromati cmanifold, compared to figure 4a.

0·5 ppm in the Ile-NH signal upon Tb^{3+} addition is obvious as in the case of CD_3OD.

We thus conclude that the conformation of CLPA in these two solvents is the same *i.e.* a *ß*-turn conformation with intramolecular hydrogen bonding and a *cis* pro residue. As per earlier evidence, the hydrogen bonding appears to occur *via* the phe-3 and the Ile-7 amide hydrogens as donors. Tb^{3+} ion appears to bind weakly and the maximal spectral alterations appear to be at a mol ratio of 5:1 CLPA: $TbCl_3$ and the ion appears to be affecting the emission properties of the phe residue through a proximal energy transfer mechanism. Calcium ion does not affect the spectral properties of CLPA to the extent that the calcium in mimic Tb^{3+} does. Though *ß*-turn is evident in CLPA, strong complexation with Ca^{2+} appears absent. Its complexation with Tb^{3+} appears to be at the surface, as an outer sphere complex involving the *f* electrons of the lanthanide, as in the case of antamanide (Wieland *et al*., 1972).

Lanthanide ion translocation across liposomes by CLPA

We next explored the possibility of whether CLPA can translocate lanthanide ions across a unilamellar vesicle, or a liposome. $Pr(NO_3)_3$ was the salt chosen since (i) Pr^{3+} is a well-known mimic of Ca^{2+} ion (Mikkelson, 1976) and (ii) Pr^{3+}, being paramagnetic, would 'lanthanide-shift' the NMR signals of the outer monolayer choline headgroup nuclei at first when added to a suspension of unilamellar vesicles (ULV) of a phospholipid such as dimyristoyl phosphocholine (DMPC): a similar shifting of the inner monolayer choline headgroup NMR signals would occur only when Pr^{3+} is able to traverse across the ULV bilayer. This then offers a NMR spectral assay of the kinetics of Pr^{3+} transport across the vesicle, mediated by a transport-inducing agent. The details of such experiments have been given elsewhere (Sankaram, 1983; Sankaram *et al*., 1985; Hunt, 1980).

Such a kinetic assay on the transport of Pr^{3+} across a DMPC ULV, mediated by varying mol ratios of CLPA: DMPC revealed that the peptide is indeed able to mediate Pr^{3+} entry across the liposomes (see figure 5 for an illustrative experiment at CLPA: DMPC = 1: 17). When the data at various CLPA: DMPC mol ratios were analyzed by means of a plot of log (rate of Pr^{3+} entry) against log (CLPA concentration), we obtained a stoichiometry of the transporting species to be 4·8 ± 0·2 of CLPA per Pr^{3+} ion. That CLPA-mediated vesicular fusion does not ocuur was confirmed in experiments involving (i) the addition of CLPA to performed DMPC vesicles and (ii) the use of performed vesicles of DMPC + CLPA, and no difference in the kinetic parameters or the NMR signal strengths were seen in the two cases. It thus appears likely that CLPA can translocate Pr^{3+} ions across unilamellar vesicles, though its efficiency does not seem to compare well with those of valinomycin or A23187, the well-known ionophores (Sankaram *et al*., 1985). The comparison of CLPA is more apt to the detergents Triton X-100 or the bile salts, which are themselves no strong ion-complexones and yet show multiple stoichiometry in transporting Pr^{3+} across liposomes (Hunt, 1980; Hunt and Jawaharlal, 1980).

On the other hand, when the ability of CLPA to translocate Ca^{2+} across liposomes was tried using standard fluorescence assays (Nagaraj *et al*., 1980), we did not find any entry of external Ca^{2+} into the liposome. It thus appears that the translocation of Pr^{3+} occurs essentially through its outer sphere complexation with CLPA and a slow diffusion process (Ting *et al*., 1981). Ca^{2+} does not bind to CLPA

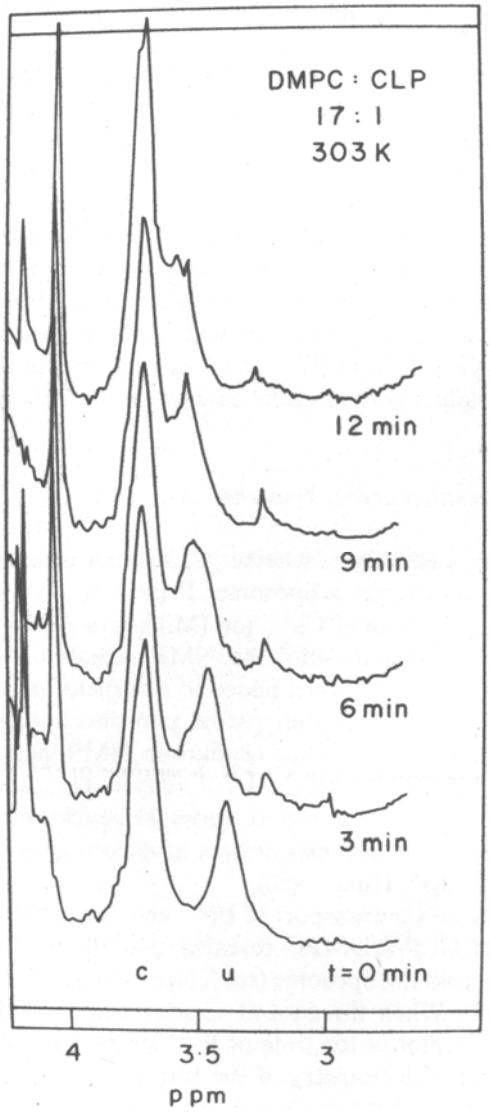


Figure 5. Time dependent changes in the NMR signals of the headgroup choline protons of DMPC in preformed ULVs of DMPC:CLPA (mol ratio 17:1) upon the addition of 10 mM P r $(NO_3)_3$ to the external medium at time t = 0. The ULVs contained 10 Mm $La(NO_3)_3$ within, in order to maintain isotonicity. The signal C refers to the outer monolayer protons that are ion-complexed and downfield shifted, while U refers to the inner headgroup protons. T = 303K, above the phase transition temperature of DMPC. The experimental details are as in Ting *et al*. (1981).

directly and neither can it form an outer sphere complex with the peptide, and thus does not get translocated by CLPA.

The effects seen with the lanthanides are thus not reflective of an ionophoretic ability of CLPA towards group I or II cations, but essentially due to the surface complexation by the lanthanide ions. In light of this, we believe that experiments

using lanthanide ions does not necessarily indicate any intrinsic Ca^{2+}-ionophoretic ability of the transporting agent but only a specialized case of outer sphere effects. Thus, it is questionable whether substances such as the bile salts, Triton X-100, cardiolipin or lysophosphatidyl choline, which are seen in NMR experiments to translocate Pr^{3+}, are true ionophores.

Note added in proof

We have since done more extensive 2D-NMR and NOE measurements on CLPA in d_6-Me_2SO at various temperatiures. This has led to some reassignments of signals and also inter-proton distance estimates in the molecule. A detailed paper on the NMR measurements, energy minimization calculations and a model for the conformation of CLPA will soon appear. We are grateful to Professors P. Balaram and C. Ramakrishnan for their interest that has led to this detailed structural analysis.

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