Acyclic model peptides with ionophoretic activity

Pr^{3+} transport by N-*t*Boc-Pro-Xxx-Ala-$NHCH_3$

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Two linear synthetic peptides, N-*t*Boc-Pro-Gly-Ala-$NHCH_3$ and N-*t*Boc-Pro-D-Ala-Ala-$NHCH_3$, have previously been shown by us to complex with Ca^{2+} and form 2:1 (peptide:calcium) complexes. Here we report their binding to Pr^{3+} and demonstrate, by ^1H NMR, the peptide-mediated transport of Pr^{3+} across dimyristoylphosphatidylcholine unilamellar vesicles via a 2:1 ion-sandwich complex.

Peptide ionophore *Ion transport* *Peptide-ion complex* *β-turn* *Model membrane* *Calcium-binding peptide*

1. INTRODUCTION

The discovery that ionophores isolated from microorganisms can induce cation translocation across model and biological membranes has led to extensive physicochemical studies dealing with the structural, kinetic and mechanistic aspects of the ionophore-mediated transmembrane cation transport and these studies, in turn, have helped in the identification of transport proteins in higher organisms [1]. An important approach in the study of ion transport across membranes is the use of synthetic molecules that possess that ionophoretic activity. Several synthetic cyclic peptides have been reported to mimic the natural ionophores [2,3].

We have been interested in the study of the functional characteristics of the β-turn which is an important secondary structural feature in globular proteins. Using synthetic model peptides, we have shown that the β-turn is essential for the enzymic hydroxylation of proline residues in the nascent collagen molecule [4,5]. Very recently, we have shown that the linear tetrapeptides, N-*t*Boc-Pro-Gly-Ala-$NHCH_3$ and N-*t*Boc-Pro-D-Ala-Ala-$NHCH_3$, can selectively bind Ca^{2+} in solution [6,7]. The initial conformation of these peptides has been found to be a consecutive or double β-turn which undergoes a rearrangement upon calcium binding. These results have prompted us to investigate whether acyclic peptides are capable of transporting metal ions across model membranes. Our results reported here show that these peptides do act as ionophores for the transport of Pr^{3+}. To our knowledge, this is the first attempt demonstrating the ionophoretic activity of small acyclic model peptides.

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2. MATERIALS AND METHODS

Synthesis and characterization of the peptides N-*t*Boc-Pro-Gly-Ala-$NHCH_3$ and N-*t*Boc-Pro-D-Ala-Ala-$NHCH_3$ have been described [7]. L-α-Dimyristoylphosphatidylcholine (DMPC) and the deuterated solvents (D_2O and methanol-d_4) were obtained from Sigma and Stohler, respectively. $La(NO_3)_3$,$Pr(NO_3)_3$ and $Pr(ClO_4)_3$ and $Pr(ClO_4)_3$ were prepared from their respective oxides (obtained from Alfa Inorganics) by treatment with concentrated nitric acid and recrystallization from double-distilled water. Acetonitrile (99.9% pure) used in CD studies was obtained from Fluka and distilled over CaH_2 before use.

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The CD spectra were recorded on a Jasco J500 spectropolarimeter calibrated with a standard solution of d_{10}-camphorsulphonic acid. Details of vesicle preparation for the transport kinetics experiments and data analysis have been presented in [8–10]. The concentration of the lipid used for NMR experiments was 18 mM and the peptides were added as solutions in methanol-d_4 to yield the required lipid:peptide ratios. ^1H NMR spectra were recorded at 40°C on a Varian FT-80A NMR spectrometer equipped with a variable-temperature accessory.

3. RESULTS AND DISCUSSION

The ability of the Gly and D-Ala peptides to bind Ca^{2+} selectively has been demonstrated by us in [6,7]. Our analysis of the CD titration data for a multiple complex equilibrium by the Reuben method [11,12] has shown that these peptides form predominantly 2:1 (peptide:calcium) complexes. The ^1H and ^{13}C NMR experiments have revealed that coordination to Ca^{2+} occurs mainly from the carbonyl oxygens of the two peptide molecules [6,7]. The CD spectra of the D-Ala peptide in the absence and presence of $Pr(ClO_4)_3$ in acetonitrile are shown in fig.1. The changes in the CD spectrum of the peptide on Pr^{3+} addition are similar to those observed with the addition of $Ca(ClO_4)_2$ [6,7]. This could be taken to indicate that Pr^{3+} behaves very similarly to Ca^{2+} in its binding to the peptide, as has been established in a large number of cases including calcium-binding proteins [13]. Similar CD data have also been obtained for the

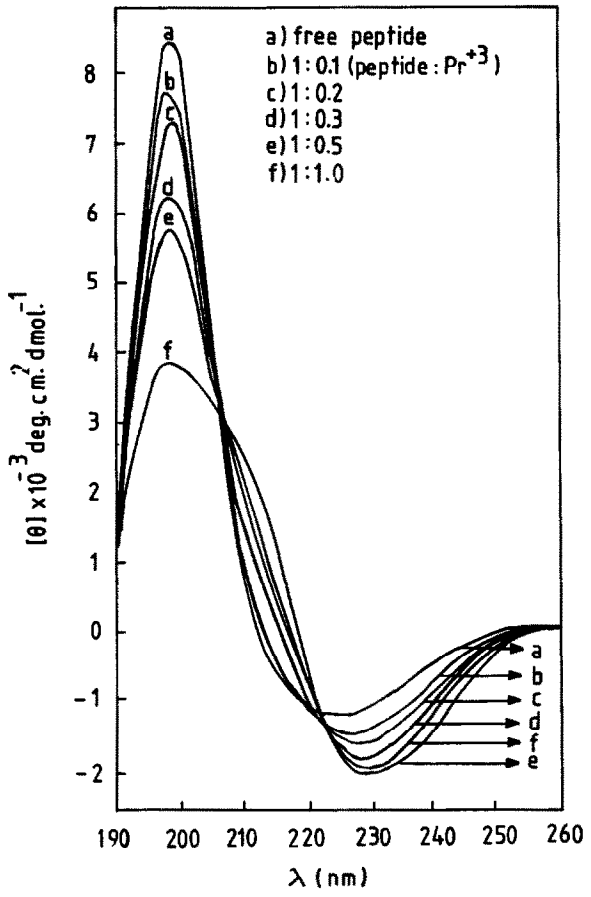

Fig.1. CD spectra of *t*Boc-Pro-D-Ala-Ala-$NHCH_3$ in acetonitrile in the absence and presence of $Pr(ClO_4)_3$. The molar ratios of Pr^{3+}:peptide are indicated on the spectra. Peptide concentration, 0.86 mM.

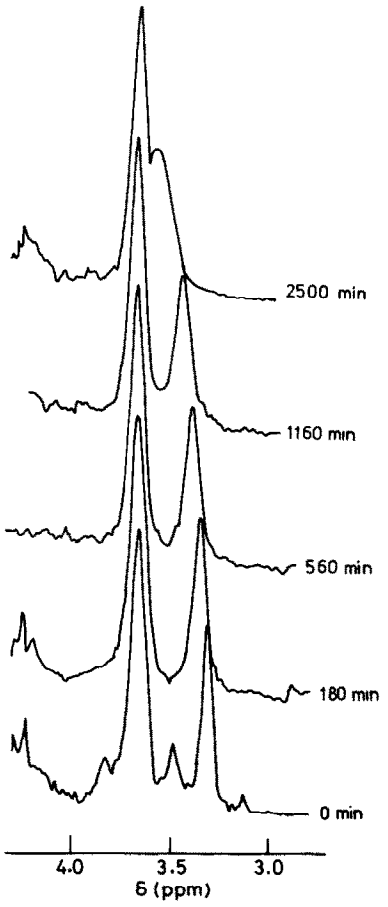

Fig.2. Time-dependent changes in the choline proton region for Pr^{3+} transport across DMPC ULVs mediated by the Gly peptide at 40°C (lipid:peptide, 50:1).

Gly peptide (not shown). Earlier analysis of the CD data has revealed that the two peptides form predominantly 2:1 complexes with Ca^{2+} and it is reasonable to assume that the same would also prevail in the case of Pr^{3+}.

The kinetics of transport of Pr^{3+} mediated by the two peptides at different lipid:peptide molar ratios (in the range 250:1–50:1) across DMPC unilamellar vesicles (ULVs) have been followed by ^1H NMR at 40°C. The vesicles contained 10 mM $La(NO_3)_3$ in the intravesicular medium and 5 mM $Pr(NO_3)_3$ plus 5 mM $La(NO_3)_3$ in the extravesicular medium. The separation between the 'outer' and 'inner' choline proton ($N(CH_3)_3$) signals induced by Pr^{3+} was found to be maximum in the absence of the peptide. The kinetics of peptide-mediated Pr^{3+} transport were followed by recording the spectra at regular intervals immediately after the addition of the peptide. The inner choline proton signal is found to shift towards the outer one with concomitant reduction in intensity and increase in linewidth, indicating that Pr^{3+} is being transported inside the vesicles with time.

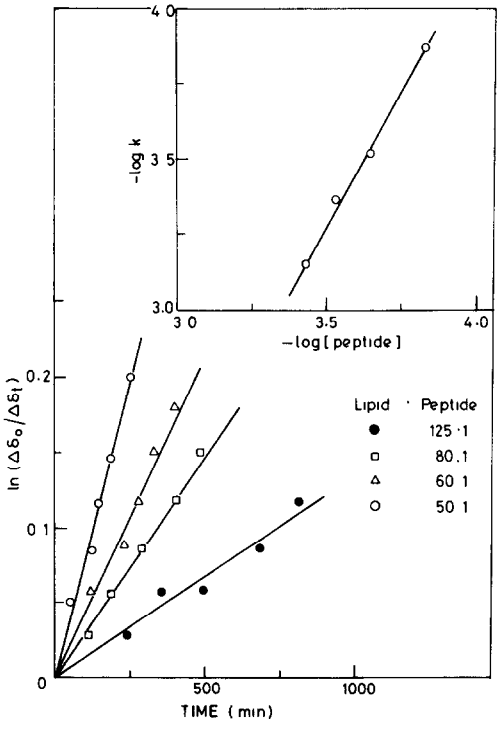

Fig.3. $\ln(\Delta\delta_0/\Delta\delta_t)$ vs time plots for DMPC–Gly peptide–Pr^{3+} system. Inset: log k vs log [peptide].

The spectra of the control samples (i.e. without the peptide) were also recorded periodically and did not show any such changes during the entire course of the kinetics experiment. The transport was considered to be over when the inner choline signal could no longer be identified. The time-dependent changes in the choline proton resonance region during Pr^{3+} transport mediated by the Gly peptide at a lipid:peptide ratio of 50:1 across DMPC ULVs at 40°C are shown in fig.2.

The initial slopes of the lines obtained by plotting $\ln(\Delta\delta_0/\Delta\delta_t)$ vs time yielded the apparent rate constants (k) at different concentrations of the peptide. A double-logarithmic plot of the peptide concentration vs the rate constants was used to calculate the stoichiometry of the transporting species [8]. Figs 3 and 4 show the $\ln(\Delta\delta_0/\Delta\delta_t)$ vs time plots for Gly and D-Ala peptides respectively, the double-logarithmic plots being shown as insets. The rate constants at different concentrations of the two peptides are given in table 1.

The results of the kinetics experiments have shown that both the Gly and D-Ala peptides permit the passage of Pr^{3+} across DMPC ULVs. The observed changes in chemical shift and linewidth

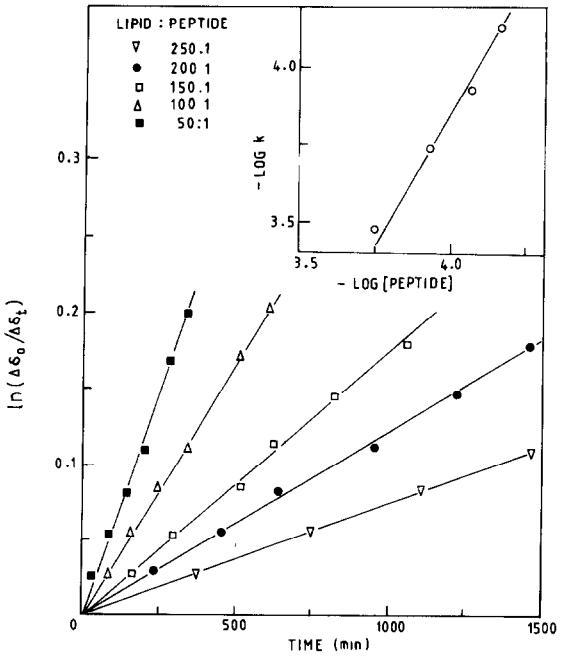

Fig.4. $\ln(\Delta\delta_0/\Delta\delta_t)$ vs time plots for DMPC–D-Ala peptide–Pr^{3+} system. Inset: log k vs log [peptide].

Table 1

Rate constants for the peptide-mediated Pr^{3+} transport across DMPC ULVs at 40°C

Peptide	Peptide (mM)	Lipid:peptide (mole ratio)	Apparent rate constant (k) ($\times 10^4$)	Slope of double-logarithmic plot
Gly	0.15	125:1	1.35	
	0.22	80:1	2.99	1.84 (± 0.10)
	0.30	60:1	4.35	
	0.37	50:1	7.09	
D-Ala	0.07	250:1	0.73	
	0.09	200:1	1.17	
	0.12	150:1	1.84	1.85 (± 0.15)
	0.18	100:1	3.31	
	0.36	50:1	11.75	

of the inner choline proton signals with time have been interpreted previously as being due to 'slow mediator exchange' and are consistent with a model in which the transmembrane ionic motions are slow on the NMR time scale [10]. The analysis of the dependence of the apparent rate constants on the peptide concentration suggests that a 2:1 'ion sandwich' complex is the transporting species for Pr^{3+} across DMPC vesicles. It is extremely interesting to note that the 2:1 complex has also been observed for the binding of Ca^{2+} by the peptides used here in acetonitrile solution [7]. Assuming the known similarity between Ca^{2+} and Pr^{3+} in their binding to peptides, it would appear that the 2:1 ion sandwich complex formed in free solution is maintained inside the membrane and is responsible for transporting the metal ion across the membrane. The conformation of the peptide in such a complex could be such that the 4 peptide carbonyl groups are coordinated to the cation [6,7]. Considering the apolar nature of the side chains in the peptide and the conformations of lasalocid-divalent cation complexes [8,14], it is reasonable to assume that, in the 2:1 complex, the two peptide molecules will be facing each other in the *anti* configuration as this gives a hydrophobic exterior and a hydrophilic interior to the complex in its entirety. The prerequisite for the formation of the complex has been shown by us to be the double β-turn, where a type II is followed by a type I' β-turn [6,7]. This conformation has been found to be more stable in the case of the D-Ala peptide when compared with the Gly peptide and the former peptide exhibits a higher binding constant than the latter [7]. This may have a bearing on the data we obtained here on the apparent rate constants for Pr^{3+} transport which show that the D-Ala peptide is a more effective (i.e. faster) mediator of transport than the glycine analog (see table 1). It is also likely that the former peptide would offer a better hydrophobic exterior compared to the latter and act as a better ion-transporting agent.

In conclusion, the present results demonstrate that small linear peptides initially having the double β-turn conformation cannot only bind to Ca^{2+} and Pr^{3+} but also induce translocation of cations across membranes in a manner similar to that of carboxylic ionophores.

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