Biophysical Chemistry 21 (1985) 211–215
Elsevier

BPC 00908

POLY(DL-PROLINE), A SYNTHETIC POLYPEPTIDE BEHAVING AS AN ION CHANNEL ACROSS BILAYER MEMBRANES

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Received 19th July 1984
Accepted 1st November 1984

Key words: Poly(DL-proline); Ion channel; Bilayer membrane; Ion transport

The synthesis and characterization of poly(DL-proline) are reported in relation with its predicted property of forming ion channels across membranes. The analysis of the conductance induced in synthetic bilayer membranes doped with poly(DL-proline) shows ionic permeoselectivity and the characteristic time course of fluctuations of ion channels, according to the similarity with the active structure of gramicidin A in membranes during the ion passage. An alternative mechanism of ion transport across bilayer membranes is also advanced.

1. Introduction

It is well known that several naturally occurring peptide and depsipeptide antibiotics activate the alkali ion permeability of artificial and biological membranes [1,2].

Intensive chemical and biophysical studies allow the recognition of two main molecular mechanisms of such transmembrane ion permeability involving two different classes of molecules: ion carriers and ion channels; the cyclododecadepsipeptide valinomycin and the pentadecapeptide gramicidin A are typical examples of the two classes, respectively. Both families of substances have sequences of amino acids (peptide), or of amino acids and hydroxy acids (depsipeptide) with regular enantiomeric alternation.

Theoretical studies showed that this distinctive feature results, for the principle of conformational equivalence (or quasi-equivalence), in a stabilization of cyclic conformations having an inner ion complexing core or, alternatively, of channel structures with pores suitable for the passage of alkali ions [3,4].

In fact, such structural features, when the substances possess side chains capable of interacting with bilayer lipidic membranes, activate the passage of ions across membranes, in the first case as anchored to liposoluble carriers and in the latter case through the hydrophilic pores acting as true ion conductors.

In spite of their different structures and conductance mechanisms the two classes of molecules are conformationally closely related. In fact, we have shown [3,4] that the cyclic conformations obtained by coupling the inversion of configuration with the inversion of conformation are generally characterized by polar cores capable of interacting with ions, whereas the side chains (nonpolar) protrude on the outside, providing the hydrophobic interactions with the lipidic phase; slight deviations from conformational equivalence, however, result in the formation of helical structures where these features are largely preserved.

The present paper illustrates the case of the alternating poly(DL-proline) synthesized in our laboratory as a model of a channel conductor, which in spite of the lack of hydrogen bonds, behaves as gramicidin A with respect to ion permeability across membranes on account of the

strict similarity of their 'active' structure during ion passage. The channel structure predicted on the basis of the conformational analysis is reported in a previous paper [4].

2. Materials and methods

NMR spectra were recorded using a Bruker 270 instrument. Infrared spectra were recorded on a Perkin Elmer IR457 spectrophotometer.

Black films were made from a 5% solution of monoolein (Sigma product) dissolved in *n*-decane (Fluka chromatography product). The bilayer membranes were formed on a 0.2 mm^2 hole in a Teflon membrane partitioning two aqueous solutions of 0.5 M KNO_3 or NaCl (Merck analytical grade); their measured electrical resistance was about 5×10^{10}–10^{11} Ω.

Deionized water, having a specific conductance of 10^{-7} Ω^{-1} cm^{-1}, was used as solvent.

Cells, similar to those employed by Eisemberg et al. [5] and a previously described apparatus [6], were used for channel conductance measurements. The applied voltage ranged between 0 and 100 mV across membranes.

Once a stationary baseline had been reached, several drops of dilute solutions of poly(DL-proline) in water, up to a final concentration in the cell of about 10^{-9} M, were added to one side of the cell. After a few minutes typical concentration-dependent channel conductances were observed.

Tests were carried out with black films doped with gramicidin A: the characteristic single channel response was obtained.

2.1. Synthesis and characterization of poly(DL-proline).

As illustrated in fig. 1, the polymer was synthesized via the pentachlorophenyl ester of the tetramer obtained from dimeric intermediates. The product was purified on a Sephadex G-10 column using 0.1 N acetic acid as eluent and lyophilized. All the intermediates of the synthesis were purified and characterized. The tetramer derivative, tBoc(L-Pro,DPro)$_2$OH, was also studied via X-ray crystal structure analysis [7].

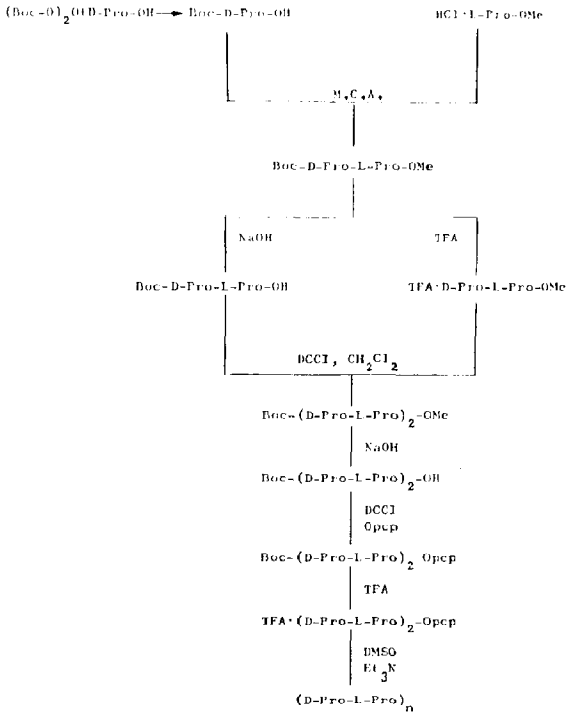

Fig. 1. Scheme of the synthesis of poly(DL-proline). Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; Opcp, pentachlorophenol; DCCI, dicyclohexylcarbodiimide; MCA, mixed carboxy anhydride.

The molecular weight was estimated by agarose gel chromatography in 6 M guanidine hydrochloride by comparison of elution peaks of the polymer with those of polypeptide molecular weight markers (molecular calibrants: myoglobin, M_r 17800; lima bean trypsin inhibitor, M_r 8400; insulin β-chain, M_r 3400; L-tryptophan, M_r 204). The elution peak indicates a molecular weight a little greater than that of the insulin β-chain. The molecular weight was then determined by ultracentrifugation in water according to the mid-point method of Yphantis [8]: the M_r value obtained was 4000, corresponding to approx. 40 amino acid residues. Elemental analysis of the sample recovered by lyophilization of the volume eluted indicated a considerable amount of water of hydration in the polymer. Poly(DL-proline) was characterized using infrared and NMR spectroscopy.

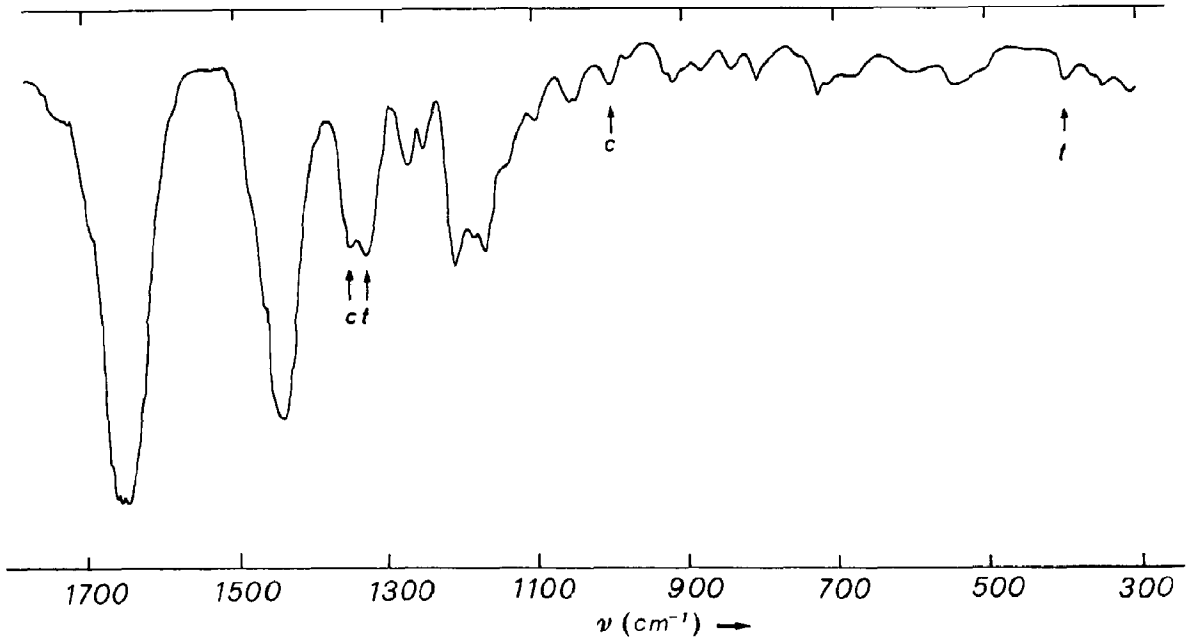

Fig. 2. Infrared spectrum of poly(DL-proline) in KBr.

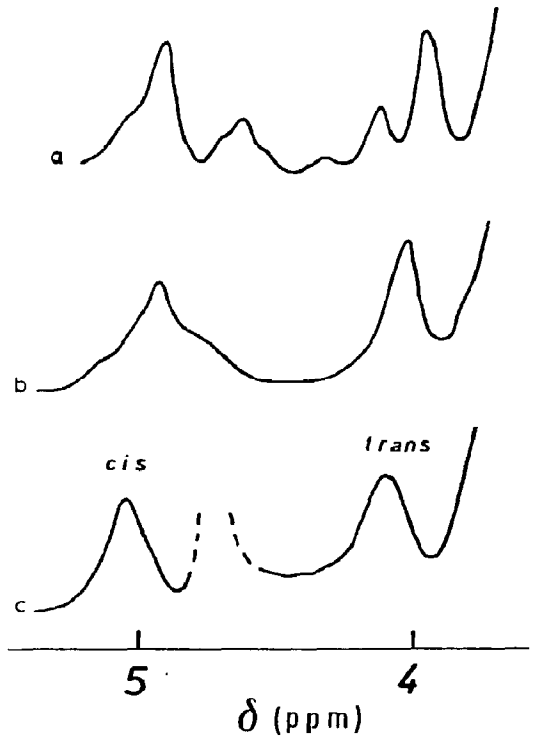

Fig. 2 shows the infrared spectrum of poly(DL-proline) in KBr. Typical bands assignable to the *cis* and the *trans* peptide conformations are detected according to Isemura et al. [9]. Fig. 3 shows the ^{1}H-NMR spectrum of the $C_\alpha H$ region in three solvents (dimethyl sulphoxide (DMSO), C^2HCl_3 and $C^2H_3O^2H$). The signals are dispersed over a wider range of chemical shifts (3.95–5.10 ppm) than for the homo-configurational poly(L-proline) I (all-*cis*) and II (all-*trans*) polymers.

In all the solvents investigated, two signals with similar integrals (at about 4 and 5 ppm) appear to account for most conformers which we assigned to the *trans* and *cis* peptide conformations, respectively. This interpretation was based on the $J_{C_\alpha H\text{-}C_\beta H_2}$ splittings in triplets and quadruplets of the signals relative to the *trans* and *cis* conformations in the tetramer according the proline ring puckers as found in the X-ray crystal structure [7]; this assignment also agrees with the NMR results

Fig. 3. ^{1}H-NMR spectra of $C_\alpha H$ region of poly(DL-proline) in (a) DMSO, (b) C^2HCl_3, (c) $C^2H_3O^2H$.

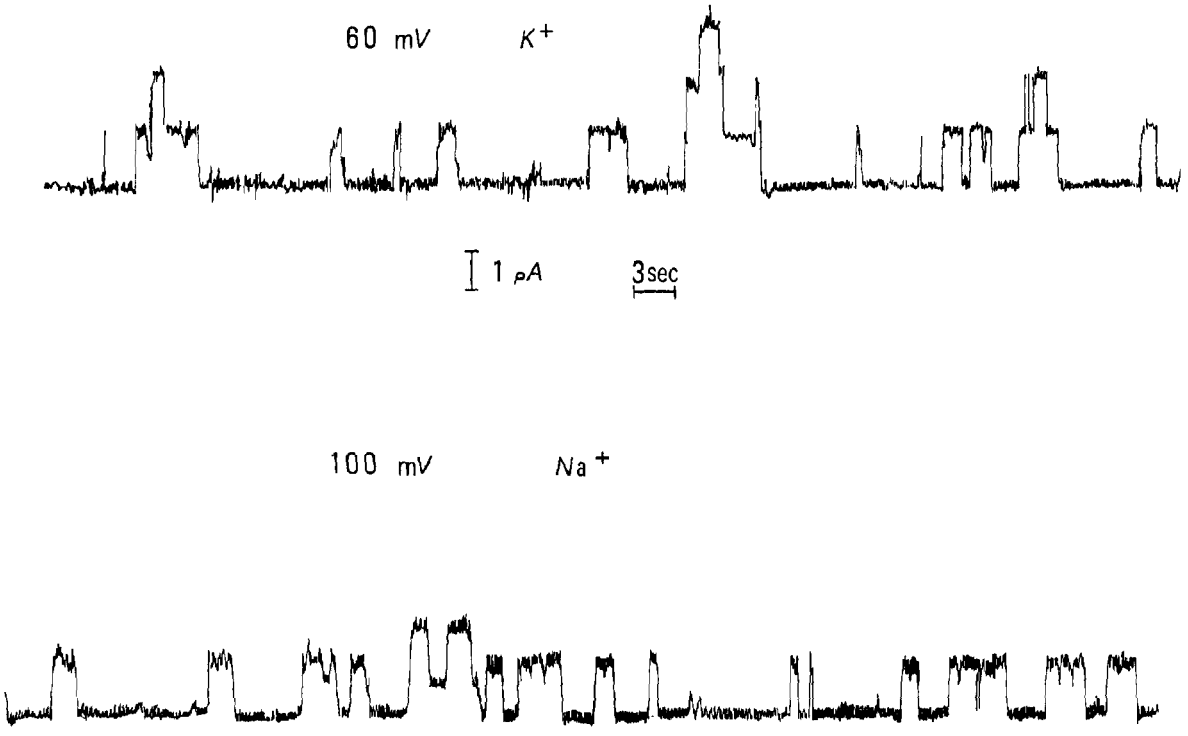

Fig. 4. Conductance fluctuations of the ionic current of a monoolein bilayer doped with poly(DL-proline) (≈ 0.001 ppm) in the case of Na^+ (a) and K^+ (b) under fixed electric potentials.

obtained in proline-containing cyclic compounds [10].

In addition to the pair of prominent signals, the spectrum in DMSO shows additional resonances, which we assigned to the differential influences of the next peptide residues on the $C_\alpha H$ signals; these further splittings appear as shoulders in the C^2HCl_3 spectrum. This indicates that poly(DL-proline) can assume different sequences of *cis* and *trans* peptide bond conformations with a preferential *cis-trans* alternation along the chain according to the theoretical predictions [4] as well as the X-ray results on the homologous tetrameric derivative [7].

However, it is plausible that the presence of ions could favour, in the ordered lipidic phase in membranes, the *trans* conformation over the all-*trans* channel structure which we have associated with the signal at 4.11 ppm in DMSO.

3. Results and discussion

As shown in a previous paper [4], poly(DL-proline) is expected to promote the conductance across bilayer membranes with the channel mechanism.

In fact, conductance measurements in black films of monoolein and lecithin show a time course of the ionic current of channel conductors typical of gramicidin A [1]. Fig. 4a illustrates recording examples of about 100 s, corresponding to a bilayer formed from a dispersion of monoolein in decane and held at 100 mV (vertical calibration bar 1.0 pA); the signal was sampled 10 times per s; the electrolyte was 0.5 M NaCl at room temperature. Similarly, fig. 4b illustrates the corresponding fluctuations of transmembrane current in the case where 0.5 M K NO_3 was used as electrolyte and with the potential fixed at 60 mV. In both cases the appearance of discrete current fluctuations

indicates single channel events; where the different unit channel conductances indicate a differential permeoselectivity for Na^+ and K^+: typical values of the most frequent single events are 13 and 25 pS, respectively, which are of the same order as that found in the same membranes doped with gramicidin A. As in the natural compound [11], anomalous channels with different conductivity were occasionally detected. In our case a plausible explanation takes into account the heterogeneity of the polypeptide molecular weight; it should be noted, however, that this dispersion is not so critical because the polymer is amphiphilic and the excess of the chain with respect to the thickness of the membrane could be dissolved in the aqueous phases.

This pattern of similarity is no longer valid in the case of Rb^+: in contrast to gramicidin A where the permeoselectivity of Rb^+ is greater than that of K^+ (50 vs. 29 pS), in the case of poly(DL-proline), the unit conductance of Rb^+ falls to a value of 10 pS, less than that of Na^+. This behaviour accounts for the poly(DL-proline) pore dimensions being smaller than in gramicidin A.

Conductance measurements attempted with Ca^{2+} were unsuccessful, indicating that the molecular mechanisms of such transmembrane conductance are inactive in the case of divalent ions.

It is interesting to note that different polypeptides and oligopeptides were shown to induce pore conductance when incorporated in black films [11–15], raising some doubts as to the generality of the channel model [15].

In fact, in our case, in spite of the similarity with gramicidin A in terms of active conformation and behaviour in membranes, it is possible to put forward a different conductance mechanism which can be considered as intermediate between the channel conductors and ion carriers and, in some respects, closer to the plausible conductance mechanism of transmembrane proteins. The poly(DL-proline) chain in the lipidic phase can in fact assume non-regular conformations, resulting in sequences of ion complexing sites, where the peptide groups are in the *trans* conformation as in cyclohexaproline, separated by more extended chain conformations where there are alternating *trans* and *cis* conformations of the peptide groups [4]. It should be noted that this is in fact the conformation of the tetramer as found by X-ray crystallography [7].

Thus, a sequence of such sites should represent the stroboscopic image of an ion shuttle carrier in the membrane. The constant value of the unit conductance should be connected with the control by the electric field on the time and space distribution of transient cationic polycomplexes into the membrane during the time constant of the measurement.

Finally, it is noteworthy that poly(DL-proline) is a polar chain which could give rise to relaxation and asymmetry effects under variable electric potential. We are now investigating this possibility.

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