Biophysical Chemistry, 31 (1988) 3–9
Elsevier

BPC 01277

A study of the conformational equilibrium of DL-oligophenylalanines in nonpolar solvents in the presence and absence of lipids by high-performance liquid chromatography

M.C. Bañó [a], L. Braco [a], B. Celda [b] and C. Abad [a]

[a] *Departament de Bioquímica i Biología Molecular and* [b] *Departament de Química Física, Facultades de Ciencias, Universitat de Valencia, 46100 Burjassot, Valencia, Spain*

Received 5 September 1987
Accepted 1 February 1988

Conformational equilibrium; DL-Oligophenylalanine; HPLC; Lipid effect

The usefulness of high-performance size-exclusion liquid chromatography (HPSEC) for the separation of dimeric and monomeric species of DL-alternating oligophenylalanines is demonstrated for the first time. The experimental data obtained as a function of time fit a simple dimer-monomer equilibrium in a nonpolar solvent such as tetrahydrofuran. A higher extent of monomerization and a decrease in the time required for reaching equilibrium were detected in the presence of either water or phosphatidylcholine. The analysis of the relative proportions of the two separated species under equilibrium conditions has allowed the influence of the oligopeptide chain length on the stability of dimeric species to be determined. The advantages of this methodology, in combination with spectroscopic techniques, in studies on autoassociating peptides are considered.

1. Introduction

Several naturally occurring peptides with alternating configurations are capable of selectively increasing the ion permeability of artificial and biological membranes [1,2]. In this connection, attention has been directed in the last few years to the synthesis of polypeptides with the ability to form ion channels [3–7].

At present, the molecular conformations of these peptides are deduced from crystal structures [3] and by using spectroscopic techniques, both in organic solvents [4–7] and upon binding to phospholipid membranes [6,7]. In solution the occurrence of interconverting conformational species in either the same or different aggregation states is very common [4–7], the contribution of each species to the total spectrum being sometimes difficult to assess.

The usefulness of high-performance size-exclusion chromatography (HPSEC) for resolving and characterizing aggregation states of autoassociating peptides in organic solvents has recently been described in our laboratory [8]. As an example, the dimer-monomer conformational equilibrium of gramicidin A (GA) (a linear hydrophobic DL-alternating pentadecapeptide), has been studied by directly visualizing the two separated species and their relative proportions under a set of experimental conditions [9,10].

We report here the application of this methodology to the investigation of the aggregation of a series of synthetic DL-alternating oligophenylalanines with terminal butyloxycarbonyl and methoxyl groups, varying in the number of residues from 8 to 15. The pentadecapeptide has a chain length similar to that of GA. The results obtained in a nonpolar solvent such as tetrahydro-

Correspondence address: C. Abad, Departament de Bioquímica i Biología Molecular, Facultades de Ciencias, Universitat de Valencia, 46100 Burjassot, Valencia, Spain.

furan (THF) are analyzed in all cases in terms of dimeric and monomeric species. The good agreement obtained from comparison of the chromatographic data with those reported from ^{1}H-NMR techniques in lipophilic solvents on the conformational tendencies of these peptides [5] demonstrates the usefulness of HPSEC as a simple, rapid and accurate procedure for the quantitative analysis of aggregation states of both natural and synthetic autoassociating peptides.

In addition, emphasis is placed on the advantages that the proposed chromatographic approach can offer to structural researchers over spectroscopic techniques. Knowledge of the relative amounts of different autoassociation states of a given polypeptide can provide valuable information for the interpretation of IR, CD and NMR spectra.

2. Experimental

2.1. Materials

DL-alternating oligophenylalanines were a generous gift from Professor Lorenzi (Institut für Polymere, ETH, Zürich). The samples studied were members VIII, IX, X, XIII and XV of the series Boc-(L-Phe)$_m$-(D-Phe-L-Phe)$_{(n-m)/2}$-OMe (n = number of residues in the oligopeptide; $m = 0$ or 1).

GA was supplied by Koch Light Labs. and was used without further purification. Egg yolk phosphatidylcholine (PC) was purchased from Merck and purified according to the column chromatographic method of Singleton et al. [11]. Chromatographic grade water and spectrograde THF were purchased from Merck. All chromatographic solvents were degassed and clarified by passing under vacuum through a 0.45 μm Micro Filtration Systems cellulose filter regenerated before use.

2.2. Methods

The liquid chromatograph consisted of an M-45 solvent delivery system and U6K universal injector from Waters Associates. The detector used was a Varian variable-wavelength ultraviolet-visible detector, set at 294 nm for GA and 260 nm for oligophenylalanines. The system was equipped with an Ultrastyragel 1000 Å column (30 × 0.78 cm inner diameter) from Waters Associates. The chromatograms were recorded with a Yokogawa Electric Works dual-channel recorder.

Oligophenylalanines and GA samples were dissolved in pure THF and aliquots taken at different times and injected. Water aliquots were added in all cases to a freshly prepared solution of the polypeptide in pure THF, so that the final peptide concentration was always 0.2 mg/ml. For experiments in the presence of PC, aliquots up to 70 μl from a highly concentrated stock solution of PC in THF (300 mg/ml) were added to the corresponding polypeptide solution in pure THF, according to the procedure previously described for gramicidin [9]. Zero time for all kinetic measurements was taken immediately after addition of the corresponding water or PC aliquot.

Chromatographic experiments were conducted at room temperature, and the column eluted isocratically with pure THF at a flow rate of 1.0 ml/min. The injection volume was 100 μl. Other details of the experimental conditions used can be found in the appropriate figure legends.

3. Results and discussion

Fig. 1 depicts, as an example, the elution profiles of samples of DL-alternating oligophenylalanines of different chain length in THF/water 92 : 8 (v/v), obtained after equilibrium was reached (3 days). The concentration of injected peptide was always 0.2 mg/ml. The shorter oligopeptides, the octa- and nonapeptides, exhibit only one peak whereas two well-resolved peaks are detected for the longer members of the series (X, XIII and XV in fig. 1). A chromatogram of GA in pure THF has been included for comparison. The values of the elution volumes found for the pentadecapeptide, XV, were virtually identical to those previously reported in THF for the double-helical dimeric ($V_e = 7.9$ ml) and single monomeric ($V_e = 8.4$ ml) conformational species of GA, using an Ultrastyragel 1000 Å column [8]. This indicates that the observed peaks could be ascribed to dimeric and

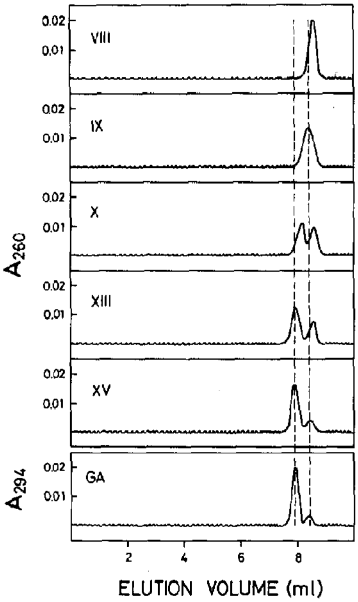

Fig. 1. Elution profiles of members VIII–X (n = 8–10), XIII (n = 13) and XV (n = 15) of the series Boc-(L-Phe)$_m$-(D-Phe-L-Phe)$_{(n-m)/2}$-OMe (n = number of residues in the oligopeptide; m = 0 or 1) in THF/water (92:8, v/v). The column (Ultrastyragel 1000 Å, Waters Associates) was isocratically eluted with pure THF at a flow rate of 1.0 ml/min. Peptide concentration was 0.2 mg/ml and the injection volume 100 μl in all cases. Samples were monitored by the absorbance at 260 nm. The elution profile for a 0.2 mg/ml GA sample in pure THF has been included for comparison. The injection volume was 5 μl.

monomeric species. This is supported by ^{1}H-NMR data, showing that oligophenylalanines containing the bulky Boc group as the N-terminus show a very strong preference for an antiparallel dimer in nonpolar solvents [5]. Moreover, when the pentadecapeptide was lyophilized from a dimethyl sulfoxide (DMSO) solution (condition under which GA has been reported to be totally monomerized [8,12]) and chromatographed in THF immediately after dissolution, only one peak, at an elution volume of 8.4 ml, was observed. This slowly converts into dimeric species at room temperature.

The existence of conformational species in a higher aggregation state, e.g., tetramers, seems very unlikely based on data reported by Lorenzi et al. [5] and on the fact that such species have been described in very nonpolar solvents (carbon tetrachloride or cyclohexane) only if the N-terminus is unprotected [4].

Several changes are observed in the elution profiles when the chain length of the injected polypeptide decreases. Firstly, although two species (dimer and monomer) are detected for XIII and X, the dimer/monomer ratio varies with the number of residues of the oligopeptide. The shorter the chain length, the higher is the monomer concentration. On the other hand, a slight progressive displacement in elution volumes towards higher values is observed in relation to XV, due to the lower molecular weights of both dimeric and monomeric species. This behaviour is expected because the chromatographic separation is based on the molecular size.

In equilibrated solutions of the octapeptide, at the same concentration, only one peak is observed (fig. 1, VIII). This can be ascribed to a monomeric species based on the following considerations: (i) The above observation that the monomer concentration increases as the chain length decreases (see fig. 1). (ii) In freshly prepared solutions of samples obtained by lyophilization from DMSO, only one peak at the same elution volume is present. In contrast with the pentadecapeptide a time dependence was not noted. (iii) The effect of the addition of either water or PC to the peptide solution did not cause any significant change in the chromatograms, as will be discussed later.

The dissociation of GA in THF has been reported to be a very slow process [8,12] and similar behaviour could be expected for the oligophenylalanines [5]. Thus, the strategy previously reported to accelerate the conformational transition of GA in THF, consisting of the addition of a small percentage of water [10], has been used in the present work. Another advantage of this approach is that a higher percentage of monomeric form is present at equilibrium [10], which allows more accurate quantitation of the monomer concentration, especially in the case of the pentadecapeptide (XV). On the other hand, it has been suggested from NMR studies that the presence of water in the deuterated solvents could be responsible for some differences in the spectral parameters [13].

Although the results presented in fig. 1 correspond to equilibrium conditions in THF/water

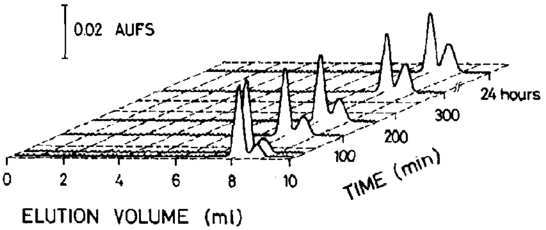

Fig. 2. Elution profiles of a 0.2 mg/ml Boc-L-Phe-(D-Phe-L-Phe)$_6$-OMe (XIII) sample in THF/water (96 : 4, v/v) as a function of incubation time. Column, Ultrastyragel 1000 Å; eluent, pure THF; flow rate, 1.0 ml/min. Samples were monitored by the absorbance at 260 nm.

(92 : 8, v/v), kinetic experiments were carried out both in THF and at different water/THF compositions. As an example, fig. 2 shows the elution profiles of XIII in THF/H_2O (96 : 4, v/v) as a function of time. Similar chromatograms were obtained for X and XV. A dimer → monomer transition was observed for the three peptides, both in THF and in the presence of water. However, the poor resolution of IX precludes the characterization of this sample (see fig. 1).

Fig. 3 shows the kinetic profiles of the monomerization of XIII (continuous curve) and GA (broken curve) in THF/H_2O (96 : 4, v/v) at 0.2 mg/ml peptide concentration, expressed as the variation of the mass fraction of monomer with incubation time. It is evident that in both cases, the elapsed time results in a displacement of equilibrium towards monomeric forms. Similar kinetic profiles were observed at the different water compositions assayed. For a given polypeptide, the higher the water content, the higher are both monomerization rate and monomer concentration.

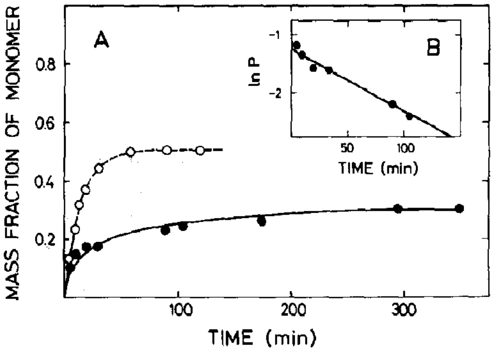

Fig. 3. (A) Solid curve: HPLC kinetic profile of Boc-L-Phe-(D-Phe-L-Phe)$_6$-OMe (XIII) monomerization in THF/water (96 : 4, v/v). The mass fraction of monomer, calculated from the heights of peaks (e.g., fig. 2), is plotted vs. the time at which each aliquot was taken. (- - - - -) HPLC kinetic profile of GA monomerization in THF/water (96 : 4, v/v). Peptide concentration was 0.2 mg/ml in both cases. (B) Plot of ln$P$ vs. time for the DL-oligophenylalanine using the data in panel A (———).

Using the results of fig. 3, a detailed kinetic analysis has been carried out to verify the existence of a simple dimer-monomer equilibrium. The procedure used has been described in detail previously [9]. In a simplified manner, for a dimer-monomer equilibrium in a given solvent:

$$M_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 2M \qquad (1)$$

an integrated form of the velocity equation can be written:

$$\ln\left(([M_2] - [M_2]_e)/\{[M_2]_0/[M_2]_e([M_2]_0 - [M_2]_e) + ([M_2]_0 - [M_2])\}\right) = \ln([M_2]_e/[M_2]_0) - \{([M_2]_0 + [M_2]_e)/([M_2]_0 - [M_2]_e)\} k_1 t \qquad (2)$$

where $[M_2]_0$, $[M_2]_e$ and $[M_2]$ refer to dimer concentration at zero time, equilibrium and a given time, t, respectively. k_1 denotes the rate constant of the direct process. For simplicity, the whole fraction of the first member will be denoted P. Fig. 3B plots, as an example, the chromatographic results in fig. 3A according to eq. 2. A good fit is observed, corroborating the validity of the model in eq. 1.

The chromatographic results obtained under equilibrium conditions for peptides X, XIII and XV at different water compositions are shown in fig. 4 (continuous curves). A similar water-induced monomerization occurs for the three oligopeptides. For a given percentage of water, the mass fraction of monomer always decreases as the peptide chain length increases. This contrasts with the more drastic monomerization of GA under the same experimental conditions (fig. 4, broken curve). Therefore, these results clearly demonstrate that the stability of the dimeric species depends not only on the amino acid composition

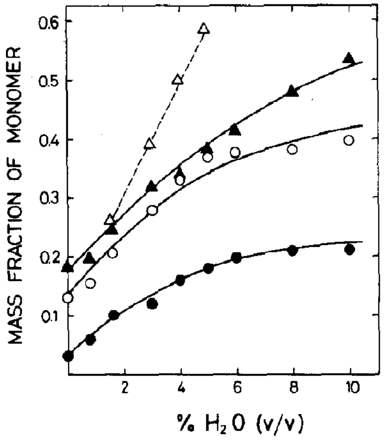

Fig. 4. (———) Effect of the addition of water on the mass fraction of monomer for DL-oligophenylalanines with differing numbers of residues. (▲) Boc-(D-Phe-L-Phe)$_5$-OMe, X; (○) Boc-L-Phe-(D-Phe-L-Phe)$_6$-OMe, XIII; (●) Boc-L-Phe-(D-Phe-L-Phe)$_7$-OMe, XV. (- - - - -) Effect of addition of water on the mass fraction of monomer for GA. The samples were analyzed after equilibrium was reached (3 days for phenylalanines; 24 h for GA).

of the peptide but also on the chain length of the oligopeptide. The addition of a small amount of water influences the peptide dimer-monomer conformational equilibrium. This can be correlated with the spectral changes described from NMR techniques [13].

A detailed picture of these systems, in terms of different aggregation states, may be elucidated by the precise chromatographic analysis of the sample at each moment. In addition, it must be emphasized that the chromatographic determination of monomer and dimer concentrations at equilibrium provides a rapid and accurate method for the evaluation of the dissociation constant, K_d [8]. Table 1 summarizes the K_d values as a function of water content, for oligophenylalanines X, XIII, XV and GA.

Concerning the effect of phospholipid, it has been reported that PC induces GA monomerization and that this phenomenon is a function of time, lipid concentration and the molar ratio of PC to GA [8,9]. Knowledge of the relative proportion of dimeric and monomeric species in the presence of lipid can be of interest when the oligopeptide is incorporated into artificial vesicles.

Table 1

Values for the dissociation constant, K_d, of oligophenylalanines of different chain length and GA in THF/H_2O mixtures

% H_2O (v/v)	$K_d(\times 10^5)$ (mol/l)			
	XV	XIII	X	GA
0	0.02	0.4	1.1	0.85
0.8	0.1	0.6	1.3	–
1.6	0.2	1.1	2.2	2.0
3.0	0.3	2.4	4.2	5.5
4.0	0.6	3.6	4.6	11.1
5.0	0.9	4.6	6.6	18.2
6.0	0.9	4.8	8.7	–
8.0	1.1	5.0	12.3	–
10.0	1.1	5.4	17.1	–

In fact, recent chromatographic experiments with GA-containing liposomes revealed the importance of the previous treatment in organic solution.

Fig. 5A shows, as an example, the mass fraction of monomer as a function of time for peptide X in the presence of PC at a concentration of 2 mg/ml. The mechanism involved in the elution process has been previously discussed for GA [9]. The results in fig. 5A indicate that the conformational equilibrium of the decapeptide is very sensitive to the presence of PC. On the other hand, when the data in fig. 5A are plotted according to eq. 2 (see fig. 5B) a good fit is observed, which suggests that

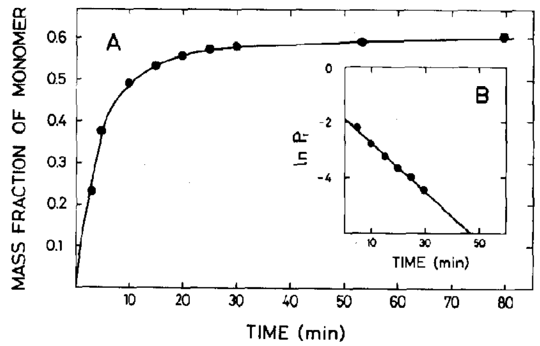

Fig. 5. (A) HPLC kinetic profile of BoC-(D-Phe-L-Phe)$_5$-OMe (X) monomerization in THF solution in the presence of PC at a concentration of 2 mg/ml. Peptide concentration was 0.2 mg/ml. (B) Plot of $\ln P_t$ vs. time according to eq. 2 using the data in panel A. P_t has been previously defined [9].

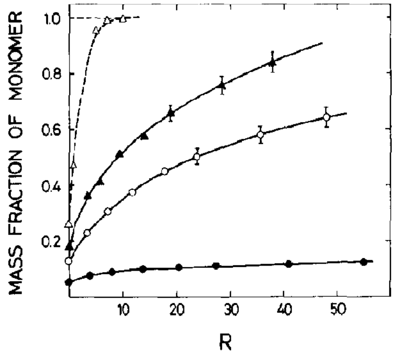

Fig. 6. Effect of PC/oligopeptide mole ratio, *R*, on the monomer mass fraction for oligophenylalanines (———) and GA (-----). Measurements were carried out at equilibrium (3 days of incubation). (▲) Boc-(D-Phe-L-Phe)$_5$-OMe, X; (○) Boc-L-Phe-(D-Phe-L-Phe)$_6$-OMe, XIII; (●) Boc-L-Phe-(D-Phe-L-Phe)$_7$-OMe, XV.

the reaction mechanism in the presence of PC is similar to that reported for gramicidin [9].

In fig. 6, the mass fraction of monomer is plotted vs. the phospholipid/polypeptide molar ratio, *R*, for oligophenylalanines X, XIII, and XV (continuous curves). The results obtained for GA at 0.2 mg/ml have been included for comparison (broken curve). All samples were chromatographed after equilibrium had been reached. A lipid-induced monomerization is observed in all cases, becoming more pronounced as the chain length of the oligopeptide decreases. However, the dimeric species of peptide XV is practically unaffected by PC, which contrasts with the behavior observed for members XIII and X of the same series. The influence of the nature of the side chains is clearly evident when comparing GA and the pentadecapeptide XV. For example, for an *R* value of 10, the extent of monomerization is 100% for GA, and only 10% in the case of XV. This observation is of particular importance for further studies on the ion permeability of lipid bilayers containing oligophenylalanine XV.

In conclusion, the HPSEC methodology used has allowed for the first time separation of dimeric and monomeric species of a series of oligophenylalanines in THF solution. An important advantage of this technique is that the dimer and monomer concentrations can be directly determined from the chromatograms at any given time, *t*. Thus, the influence of oligopeptide chain length on the stability of dimeric species has been evaluated by analyzing the dimer → monomer conformational equilibrium in each case.

In general, this methodology may be of interest in studies of autoassociating peptides in combination with spectroscopic techniques. Characterization of the system by HPSEC makes it possible to examine previously observed spectral changes by knowing the polypeptide aggregation states. Also, direct quantitation of the conformational species by HPLC makes the mathematical treatment of the spectrum unnecessary when more than one aggregation state is present. Likewise, this method facilitates selection of the experimental conditions where only a given associated state is present in solution.

In summary, the combination of chromatographic and spectroscopic techniques could provide a more complete understanding of the structural features of the conformational species of autoassociating polypeptides in organic solution. Indeed, we have recently proposed a method for the determination of the relative quantum yields of the individual conformational species of gramicidin based on the fitting of the experimental results obtained independently from fluorescence emission spectroscopy and HPSEC [10].

Acknowledgements

We are grateful to Professor Gian Paolo Lorenzi for the oligophenylalanine samples. This work was supported by grant no. 0598/84 from CAICYT.

References

1 S.B. Hladky and D.A. Haydon, Biochim. Biophys. Acta 274 (1972) 294.
2 B.C. Pressman, Annu. Rev. Biochem. 45 (1976) 501.
3 M. Colapietro, P. de Santis, A. Palleschi and R. Spagna, Biopolymers 25 (1986) 2227.
4 G.P. Lorenzi, C. Gerber and H. Jackle, Biopolymers 23 (1984) 1905.
5 G.P. Lorenzi, C. Gerber and H. Jackle, Macromolecules 18 (1985) 154.

6 T. Higashijima, T. Miyazawa, M. Kawai and V. Nagai, Biopolymers 25 (1986) 2295.

7 J. Kamegai, S. Kimura and Y. Imanishi, Biophys. J. 49 (1986) 1101.

8 L. Braco, C. Abad, A. Campos and J.E. Figueruelo, J. Chromtogr. 353 (1986) 181.

9 L. Braco, M.C. Bañó, F. Chillaron and C. Abad, Biophys. Chem. 25 (1986) 297.

10 L. Braco, F. Chillaron, M.C. Bañó, M. de la Guardia and C. Abad, Spectrochim. Acta 43 A (1987) 1365.

11 W.S. Singleton, M.S. Gray, M.L. Brown and J.C. White, J. Am. Chem. Soc. 92 (1965) 53.

12 W.R. Veatch, E.T. Fossel and E.R. Blout, Biochemistry 13 (1974) 5249.

13 G.P. Lorenzi, H. Jackle, L. Tomasic, V. Rizzo and C. Pedone, J. Am. Chem. Soc. 104 (1982) 1728.