FEBS Letters 349 (1994) 380–384

FEBS 14322

Influence of the conformation of a macromolecule on the generation of T-cell proliferative response

A study with model polypeptides

E. Lacassie, A. Delmas*, Y. Trudelle

Centre de Biophysique Moléculaire, CNRS, 1A avenue de la Recherche Scientifique, 45071 Orléans cédex 2, France

Received 18 May 1994; revised version received 14 June 1994

Abstract

To study the influence of the conformation of polypeptidic macromolecules on the generation of T-cell epitopes, sequential polypeptides with an octamer repeat unit were designed and synthesized. They adopt mainly unordered and α-helical conformations. Among these polypeptides, those containing proline are fully or partly unordered, and are more effective at inducing T-cell proliferation than a proline-free very stable α-helical polypeptide. This extremely stable α-helical conformation, probably stabilized by aggregation, would enhance its stability against proteolytic processing.

Key words: T-cell epitope; Polypeptide; Conformation; α-Helix; Aggregation

1. Introduction

Short synthetic peptide immunogens are able to elicit a strong immune response only when attached to a carrier protein [1]. Previous studies have demonstrated that the induction of antibody responses against an haptenic determinant is mediated through cognate interaction between hapten-specific B cells and carrier-specific helper T-cells [2]. To enlist T-cell help most short synthetic peptides have, therefore, to be conjugated to a larger protein carrier providing the necessary T-cell epitopes. T-cell epitope peptides are bound to the class II major histocompatibility molecule (MHC) in an extended conformation [3]. However, relatively little is known about the requirements that must be satisfied by a carrier for immunization before processing and binding to a class II MHC molecule. Macromolecular polypeptides offer a unique system to study such a question. Suitable models simulating proteins have been studied as part of the analysis of the immune response [4]. The multichain branched polypeptide poly-L-lysine–poly-DL-alanine–poly-(tyrosine, glutamic acid) has been extensively studied [5]. Based on this polypeptide, Hudecz and Szekerke [6] and Rajnavölgy et al. [7] developed a class of synthetic branched polypeptides and studied the relationships between their chemical structure and their carrier potency at the humoral level. To design a macromolecular carrier on a rational basis, it was interesting to investigate the influence of the conformation of the polypeptide macromolecule on the generation of T-cell epitopes.

In this paper, we describe studies at the T-cellular level with sequential polypeptides in different conformations depending upon the absence or the presence of proline in the 8 residue repeating units. Their T-cell epitope activity was estimated by proliferation assays. Circular dichroism (CD) spectroscopy was used to determine their conformation.

2. Materials and methods

2.1. Synthesis of H(Leu-Lys-Glu-Leu-Leu-Glu-Lys-Leu)$_3$OH: (Po)3

(Po)3 was synthesized by the classical solid-phase method described by Merrifield [8] using the *t*-butyloxycarbonyl strategy and a PAM resin as solid support. The side chains of glutamic acid and lysine were protected by, respectively, cyclohexyl and 2-chlorobenzyloxycarbonyl groups. The peptide was deprotected and cleaved from the resin by liquid hydrogen fluoride [9], extracted with an aqueous acetic acid solution and lyophilized. The crude material was purified by gel-permeation on Sephadex G50 with 0.1 M acetic acid as eluent. Molecular weight was verified by mass spectrometry and agreed with the expected value, $[M + H]^+$: 2919.6 (expected), 2918.1 (found).

2.2. Polypeptide synthesis

The polydisperse polyoctapeptides were obtained by polycondensation of the corresponding repeat unit. The octapeptides were synthesized by classical solution synthesis. Side chains of lysine and glutamic acid were protected by benzyloxycarbonyl and benzyl groups, respectively. These protecting groups were compatible with the *o*-nitrophenylsulphenyl group used for α-amino group protection. The C-terminal amino acid was protected in the form of the *p*-nitrophenyl ester which acts as the activating group during the polycondensation step according to the Goodman-Stueben [10] 'backing-off' procedure. The peptide chain was elongated by DCCI couplings leading to the activated derivative of the repeat unit. After each coupling step, the peptide was checked for purity by thin-layer chromatography using Merck precoated 60F 254 plates (silica gel).

Polycondensation of the three protected octapeptide *p*-nitrophenyl esters was carried out by adding TEA to a very concentrated DMF solution (0.7 M of monomer) and in the presence of a stoichiometric

*Corresponding author. Fax: (33) 38 63 15 17.

SSDI 0014-5793(94)00708-X

amount of 1-hydroxybenzotriazole (HOBt) to reduce racemization. Lysyl and glutamyl protecting groups were cleaved by hydrobromic acid in trifluoroacetic acid. The crude polymers were dialyzed against 0.01 N hydrochloric acid and water, and finally freeze-dried. The average molecular weights were estimated by viscometry in TFA [11].

2.3. Conformational and aggregation studies

CD spectra were recorded between 190 and 300 nm on a Jobin-Yvon Mark IV dichrograph. The conditions used for each polypeptide are indicated in the legend of the figures. Ellipticities [θ] are given per mean residue, in degree · cm^2/dmol.

Osmometric molecular weight determinations were carried out using a Hewlett-Packard membrane osmometer equipped with a Spectrapor 3 membrane (c.o. 3,500 Da) from Spectrum.

2.4. Assay for proliferative responses of antigen-specific T cells

Balb/c mice were injected in the hind footpads with 50 μg polypeptide, dissolved in 25 μl 0.9% NaCl, emulsified in 25 μl CFA. 10 days later, draining popliteal and/or inguinal lymph nodes were removed and a single cell suspension of T-cells prepared in RPMI 1640 (Gibco) containing 2 mM L-glutamine, antibiotics, $5 \cdot 10^{-5}$ M 2-mercaptoethanol and 10% foetal calf serum. T-cells were cultured in 0.2 ml at a final concentration of $2.5 \cdot 10^6$ cells in 96-well plates with or without antigen. After 4 days of culture (37°C, 7.5% CO_2) the cells were pulsed for 18 h with [^{3}H]thymidine and then collected on glass fiber filters with a Mash cell harvester. Incorporated radioactivity was determined by liquid scintillation counting. Results were expressed as the difference between cpm with and without antigen (Δcpm). Standard deviation of quadruplicate cultures were about 10% of the mean.

3. Results

3.1. Choice of sequences

The linear polymers were designed so as to adopt mainly unordered and α-helical conformations, with a sequence repeat unit of 8 amino acids in length. Barbier et al. [12] have shown that a strict alternation of hydrophilic (Lys-Lys) and hydrophobic (Leu-Leu) doublets induces, as a rule, the formation of an α-helix. Recently, we (unpublished results) and others [13] showed that polymers with predominant cationic groups could be toxic, while compounds with a balanced charge distribution are not. Therefore, glutamyl residues were introduced to counterbalance the positive charges of the lysyl side chains. To obtain a stable α-helix, we chose to alternate Lys-Glu and Glu-Lys hydrophilic doublets to generate i,i + 4 salt bridges between Glu and Lys side chains, which are known to have a stabilizing effect on the α-helical structure [14]. Thus, the repeat unit of 8 amino acids (P_0: LKELLEKL) was chosen. To break the α-helical conformation, one leucyl residue was replaced by an α-helix breaking prolyl residue [15,16] every 4 (P_2: PKELPEKL) and every 8 (P_1: LKELPEKL) amino acids, to obtain a polypeptide with a partial α-helical conformation. The three octapeptidic repeat units were synthesized and polymerized to obtain macromolecules of molecular weight > 10,000 Da collated in Table 1.

Table 1
Sequences of polyoctapeptides

		Mol. weight (Da)
$(P_2)n$	(Pro-Lys-Glu-Leu-Pro-Glu-Lys-Leu)n	16,800
$(P_1)n$	(Leu-Lys-Glu-Leu-Pro-Glu-Lys-Leu)n	18,400
$(P_0)n$	(Leu-Lys-Glu-Leu-Leu-Glu-Lys-Leu)n	12,120

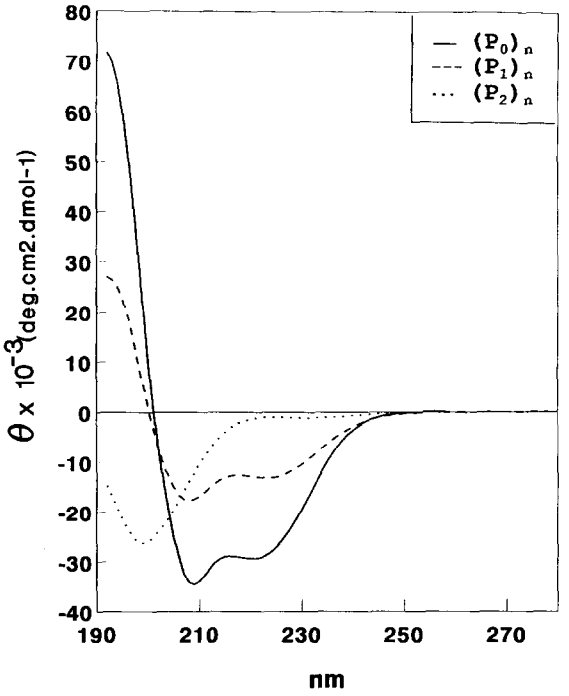

Fig. 1. Circular dichroism spectra of $(P_0)n$ at a concentration of 0.1 mg/ml in 0.001 N HCl, pH 3 (0.1 cm cell), and of $(P_1)n$ and $(P_2)n$ at a concentration of 2 mg/ml in 0.1 M phosphate buffer, pH 7.1 (0.01 cm cell).

3.2. Conformation and aggregation studies

As expected, $(P_0)n$ is α-helical, as evidenced by CD spectroscopy (Fig. 1). Owing to its rather weak solubility in water, spectra were recorded at low concentrations. Ellipticity values corresponded to a nearly fully α-helical structure (found $\theta_{222} = -30{,}000$, typically $-33{,}000$). The stability of the helical structure was investigated by varying several parameters. It appeared that pH (from 3 to 7.4), ionic strength (from pure water to 0.24 M NaF or other salts) and dilution (from $0.656 \cdot 10^{-3}$ M to $0.00656 \cdot 10^{-3}$ M, i.e. 0.1 to 0.001 mg/ml) had only a very small effect on the helical integrity of $(P_0)n$. The ellipticity could be only slightly lowered by raising the temperature to 40°C.

The polymer $(P_0)n$ was also found to aggregate. Gel-permeation chromatography using G75 Sephadex showed that the major fraction of the polymer was eluted in the void volume corresponding to a molecular weight of 70,000 Da or more. Since the molecular weight determined by viscometry in denaturating conditions (in TFA solution) was 12,120 only (Table 1), this corresponds approximately to a 6-mer aggregate. The aggregation contributes probably to the high helical stability of $(P_0)n$.

The $(P_0)3$ oligomer also showed the typical CD fea-

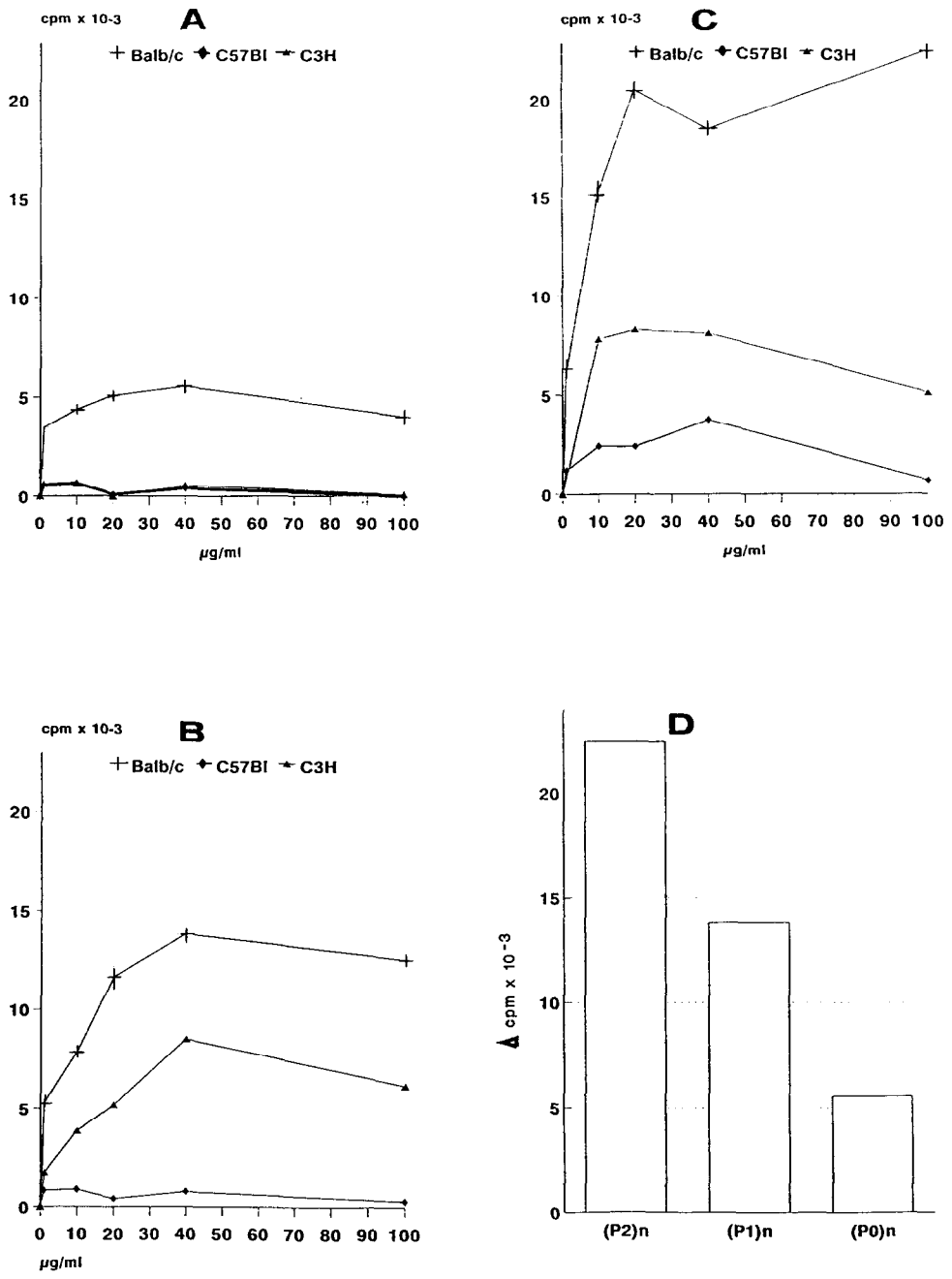

Fig. 2. Proliferative responses of polyoctapeptide-primed lymph node cells from various strains of mice. Balb/c, C57Bl/6 and C3H/j mice were immunized with 50 µg of $(P_0)n$ (A), $(P_1)n$ (B), and $(P_2)n$ (C). 10 days later, their lymph node cells were stimulated in vitro with the homologous polyoctapeptide. (D) Proliferative responses of lymph node cells of Balb/c mice to 40 µg/ml homologous polyoctapeptides $(P_0)n$, $(P_1)n$ and $(P_2)n$.

tures (not shown here) of an α-helix, although, as expected for a rather short peptide, ellipticities were lower ($\theta_{222\ nm} = -12{,}000$) than for the corresponding polymer. $(P_0)3$ is also aggregated as shown by osmometric measurements. The average molecular weight in 0.1 M NaF thus estimated corresponded to a 5-mer aggregate, consistent with the value found for the polymer.

CD spectrum of $(P_2)n$ indicates a completely unordered conformation, with a trough at 198 nm, while the CD spectrum of $(P_1)n$ with one proline per repeat unit only reflects a partly α-helical structuration which increases slightly when pH is raised from 3 to 7. Neither of the polymers aggregate as found by gel-permeation chromatography and osmometric measurements.

3.3. T-cell proliferative responses

The re-stimulation of T-cell proliferation by $(P_0)n$, $(P_1)n$ and $(P_2)n$ was systematically assayed on $H2^b$

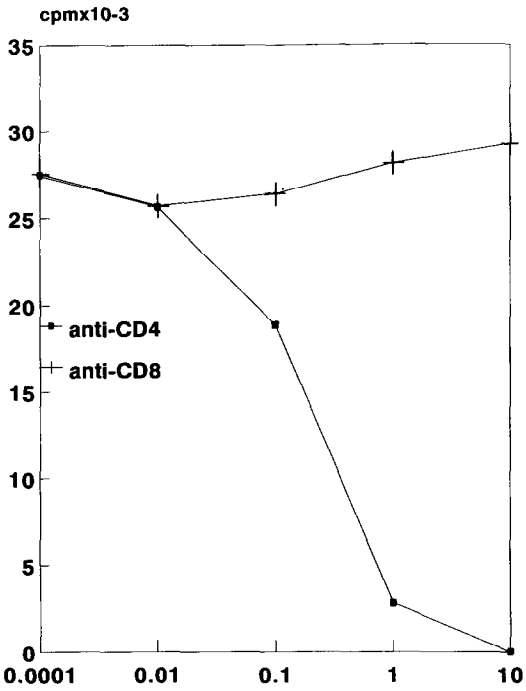

Fig. 3. $(P_2)n$-specific proliferation of $CD4^+$ T-lymphocytes. Balb/c mice were immunized with 50 μg of $(P_2)n$. 10 days later, the proliferative responses of their lymph node cells to $(P_2)n$ (40 μg/ml) were assayed in the presence of anti-CD4 or anti-CD8 monoclonal antibodies.

(C57Bl/6 mice), $H2^d$ (Balb/c mice) and $H2^k$ (C3H mice) restricted T-cells. For each polyoctapeptide, two groups of mice were immunized with either polyoctapeptide or 0.9% NaCl only, as control, to detect possible non-specific proliferation. None of these polyoctapeptides is mitogenic (data not shown). Balb/c mice show the best response after immunization with polypeptides (Fig. 2). Therefore, comparisons between T-cell activities were carried out using this strain only. Fig. 3 shows that the $(P_2)n$-specific proliferation of lymph node cells from $(P_2)n$-primed Balb/c mice was totally inhibited by the addition to the culture of anti-$CD4^+$ monoclonal antibodies but was not affected by co-culture with anti-$CD8^+$ monoclonal antibodies even at high doses. Very similar results were obtained with $(P_1)n$ as antigen. Thus, the specific proliferative response observed is due to the stimulation of the $CD4^+$ T-cell subset only.

The polyoctapeptide $(P_2)n$ induces excessive T-cell proliferation, while $(P_1)n$ (one proline every eight residues) induces a significant but less-pronounced one than that of the polymer with two prolines (Fig. 2). On $(P_1)n$-primed T-cells, the proliferation induced by $(P_2)n$ was of the same order of magnitude as that induced by $(P_1)n$, whereas on $(P_2)n$-primed T-cell, $(P_1)n$ was twofold less active than $(P_2)n$ (Fig. 4).

The polymer $(P_0)n$ (without proline), in the α-helical conformation ($\theta_{222\ nm} = -30{,}000$) is inactive (Fig. 2), whereas the oligomer $(P_0)3$, which is also helical but to a lesser extent ($\theta_{222\ nm} = -12{,}000$) is able to re-stimulate T-cell proliferation when mice received 100 μg antigen (Fig. 5). At high concentrations, $(P_0)3$ is cytotoxic, probably due to its amphipathic character [17].

4. Discussion

Our experiments aimed to study if the conformation of macromolecular polypeptides formed by tandem repeats of octapeptides could influence the generation of T-cell epitopes. Some studies regarding the conformation of T-cell epitopes by CD spectroscopy have been reported [18,19]. These studies were carried out with short peptides which usually do not adopt a well-defined conformation in water, and are forced into a more stable structure, generally α-helical, by adding trifluoroethanol. Macromolecules like sequential polypeptides of limited amino acid diversity, but of different amino acid sequences, offer a valuable system to obtain stable conformations in water. $(P_0)n$ shows a particularly stable α-helical conformation, stabilized probably by an aggregation phenomenon which was evidenced by gel-chromatography. Starting from a stable α-helical amphipathic polyoctapeptide $(P_0)n$, it was found, as expected, that progressive substitution of Leu by Pro gradually destabilizes the initial α-helix. The presence of two prolines per repeat unit in $(P_2)n$ impedes the formation of the

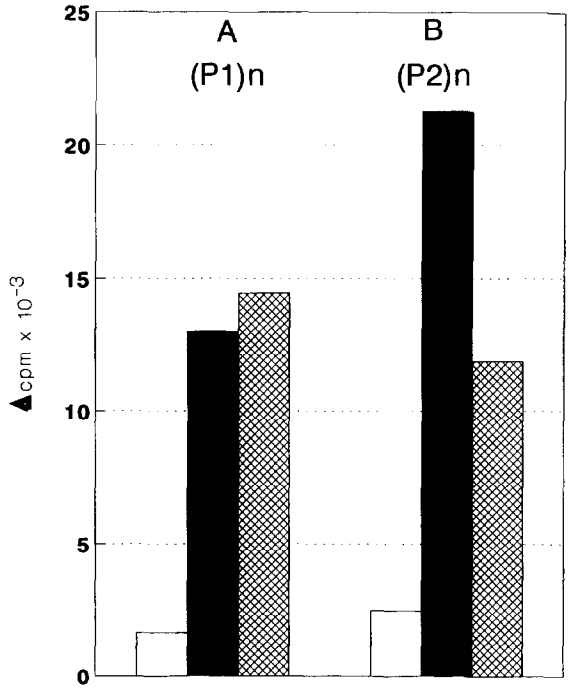

Fig. 4. Proliferative responses to heterologous polyoctapeptide. Balb/c mice were immunized with 50 μg of $(P_1)n$ (A) and $(P_2)n$ (B). 10 days later, lymph node cells were stimulated in vitro with 40 μg/ml of $(P_1)n$ (cross-hatched bars), $(P_2)n$ (filled bars) and $(P8R)n$ (open bars), an irrelevant polyoctapeptide $(KGNPREEE)n$.

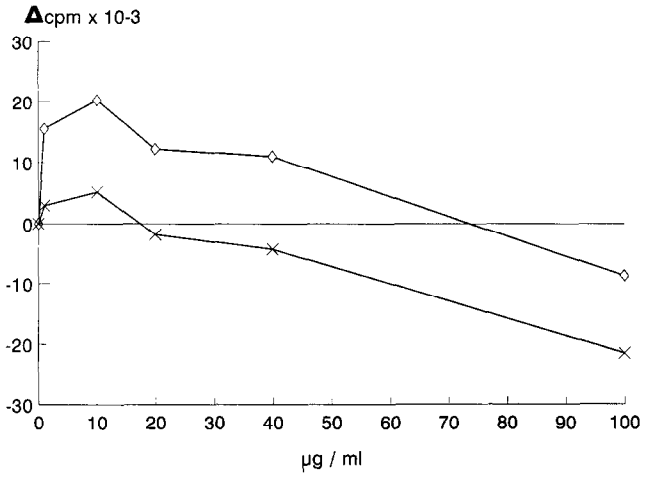

Fig. 5. Proliferative responses to $(P_0)3$. Balb/c mice were immunized with 50 μg (×) or 100 μg (◇) of $(P_0)3$. 10 days later, their lymph node cells were stimulated in vitro with $(P_0)3$.

α-helix, while $(P_1)n$, with one proline only, gives rise to a partly helical structure.

In vitro proliferation of T-cells with this series of polypeptides shows that polypeptides containing proline give rise to T-cell proliferation, while a proline-free polypeptide of very close sequence is inactive. The magnitude of the proliferative response obtained with the most active polymers is of the same order as for a natural protein such as ovalbumin [20]. The behavior of $(P_1)n$ is comparable to that of $(P_2)n$. Indeed, $(P_1)n$ induces a T-cell response that can be recalled by $(P_2)n$ within the same order of magnitude, while $(P_1)n$ recalls the T-cell response induced by $(P_2)n$ but to a lesser extent. The substitution of Leu by Pro modifies the conformation of the polyoctapeptide but also introduces a new side chain which might interact more favorably with the MHC molecules.

In the case of $(P_0)n$, several reasons can be put forward to explain the lack of T-cell response: (i) a deficiency in expressing T-cell receptors able to recognize the processed peptides; (ii) inability of MHC molecules to bind to the processed peptides and thus to present them to the T-cell receptors; (iii) the processing of the native peptide does not take place and consequently no peptides able to bind to MHC molecules are generated. Since the oligomer $(P_0)3$, which has the same sequence as $(P_0)n$ but with 3 repeat units only, re-stimulates the proliferation of T-cells, hypotheses (i) and (ii) can be ruled out so that the last one seems the more plausible. It is well-known that unordered conformations are more sensitive to proteolysis than helical and aggregated structures. For example, it has been reported that the amphipathic helical structure of two peptides analogous to β-endorphin [21,22] protects them from proteolytic degradation. In the case of amphipathic and helical P_0-based peptides, aggregation contributes probably to enhance their stability against proteolytic processing, thus rendering this macromolecule silent for the immune system.

The results herein reported illustrate the usefulness of the structural approach. Our observations suggest that conformation and aggregation must be taken into account in the study of antigen processing and presentation. Furthermore, a silent macromolecule for the immune system, like $(P_0)n$, would be of interest as a carrier of epitopes by increasing their life-time.

Acknowledgements: The authors wish to thank Mrs. J. Verhaeghe for her skillful technical assistance, Prof. G. Motta for facilities to perform proliferation assays, Dr. C. Leclerc, from Institut Pasteur Paris, for the gift of anti-CD4 and anti-CD8 monoclonal antibodies, Drs. B. Barbier and A. Brack for helpful discussions, and Dr. A. Brack for critically reading the manuscript. E.L. was supported in part by a grant from CNRS and 'La Région Centre'.

References

[1] Katz, D.H. (1980) Adv. Immunol. 29, 137–207.
[2] Mitchison, N.A. (1971) Eur. J. Immunol. 1, 10–18.
[3] Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. and Wiley, D.C. (1993) Nature 364, 33–39.
[4] Mozes, E. (1975) Immunogenetics 2, 397–410.
[5] Sela, M., Fuchs, S. and Arnon, R. (1962) Biochem. J. 8, 223–235.
[6] Hudecz, F. and Szekerke, M. (1985) Coll. Czech. Chem. Commun. Engl. Edn 50, 933–940.
[7] Rajnavölgyi, E., Hudecz, F., Mezö, G., Szekerke, M. and Gergely, J. (1986) Mol. Immunol. 23, 27–37.
[8] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[9] Tam, J.P. and Merrifield, R.B. (1985) Int. J. Peptide Protein Res. 269, 262–273.
[10] Goodman, M. and Stueben, K.C. (1959) J. Am. Chem. Soc. 81, 3980–3983.
[11] Brack, A. and Trudelle, Y. (1985) Polym. Commun. 26, 369–370.
[12] Barbier, B., Perello, M. and Brack, A. (1988) Coll. Czech. Chem. Commun. 53, 2825–2832.
[13] Hudecz, F., Gaal, D., Kurucz, I., Lanyi, A., Kovacs, A.L., Mezö, G., Rajnavölgyi, E. and Szekerke, M. (1992) J. Control. Release 19, 231–244.
[14] Marqusee, S. and Baldwin, R.L. (1987) Proc. Natl. Acad. Sci. USA 84, 8898–8902.
[15] Robson, B. and Susuki, E. (1976) J. Mol. Biol. 107, 327–356.
[16] Chou, P.Y. and Fasman, G.D. (1978) Adv. Enzymol. 47, 45–148.
[17] Reynaud, J.A., Grivet, J.P., Sy, D. and Trudelle, Y. (1993) Biochemistry 32, 4997–5008.
[18] Abergel, C., Loret, E. and Claverie, J.M. (1989) Eur. J. Immunol. 19, 1969–1972.
[19] Vita, C., Baumhüter, S. and Corradin, G. (1990) Mol. Immunol. 27, 291–295.
[20] Maloy, K.J., Donachie, A.M., O'Hagan, D.T. and Mowat, A.McI. (1994) Immunology 81, 661–667.
[21] Taylor, J.W., Miller, R.J. and Kaiser, E.T. (1982) Mol. Pharmacol. 22, 657–666.
[22] Taylor, J.W., Ostermann, D.G., Miller, R.J. and Kaiser, E.T. (1981) J. Am. Chem. Soc. 103, 6965–6966.