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Cyclolinopeptide A: inhibitor, immunosuppressor or other?‡

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INTRODUCTION

Cyclolinopeptide A (CLA), the homodetic cyclononapeptide of formula c(Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val) was first isolated in 1959 [1] from acetone extracts of linseed oil and its solid residues. Small quantities of a solid compound were purified by subsequent recrystallizations in the same solvent and characterized by means of sole chemical methods as the linear tetrapeptide H-Pro-Leu-Phe-Val-OMe.

In the 1960s, the actual chemical structure of the oligopeptide from linseed was determined [2] adopting a combination of gas chromatography of the trifluoromethyl esters of peptide fragments and mass spectrometry standard techniques. The chemical structure of CLA was then confirmed by total chemical synthesis adopting classical methods in solution. In fact, in 1964 at the 8th European Peptide Symposium, held in Noordwyk, The Netherlands, Professor F. Weygand presented the synthesis of the peptide (Figure 1), prepared through solution methods, the only way known at that time for such 'large' peptides.

In 1966, shortly before his death, Professor F. Weygand met Murray Goodman in New York and gave him a sample of the synthetic cyclic nonapeptide for stereochemical analysis. One of us (E.B.), at that time a post-doctoral student in Murray's laboratories at the Polytechnic Institute of Brooklyn, NY, in collaboration with Fred Naider, one of Murray's PhD students, undertook the examination of the conformation of the peptide by CD [3], while at the same time a NMR study [4] and conformational energy calculations [5] were undertaken by Drs Bovey, Brewster and Tonelli at the Bell Telephone Laboratories, Murray Hill, NJ. The results of these investigations were collected in the three papers appearing in the same issue of *Proc. Natl Acad. Sci. USA* in 1971 (Figure 2). Surprisingly, all the conformations proposed for the nonapeptide in solution by CD or NMR or calculated were different from the most stable conformation determined years later by our group.

In the following years, the elucidation of the 3D-structure of CLA focused the attention of researchers since its Pro-Pro-Phe-Phe sequence is shared with antamanide (AA) [6], the homodetic cyclodecapeptide c(Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-Phe) isolated from the poisonous mushroom *Amanita phalloides*. Antamanide, one of the most extensively investigated cyclic peptides, received huge scientific attention because of its strong antidote activity against phallotoxins, a family of extremely toxic bicyclic heptapeptides isolated from the same source as AA itself [7].

As a matter of fact, CLA and AA exhibit a similar biological activity. However, in contrast to AA, that — even if existing in different conformations — has been exhaustively investigated both in the solid state and in solution, the pronounced conformational freedom of CLA resulted in a discouraging difficulty in obtaining more precise information on its structural features, as demonstrated by the early studies in solution.

However, in 1968 the Polymer Research Institute at the Polytechnic Institute of Brooklyn suddenly had a grant available to us, by which, with the strong support of Murray Goodman, we had the opportunity to install and use exhaustively a x-ray single crystal diffractometer (not many of them were in circulation at that time). With this instrument we collected data on any possible peptide crystal, even if at that time the solution of the 3D-structure of an optically active compound was almost prohibitive. The diffraction data on CLA obtained at that time proved to be very valuable when in 1995, after the advent of direct methods in crystallography, we were able to solve its crystal-state structure.

The following gives a brief description of the main results obtained over the years on our understanding of the structure-activity relationships of CLA. This accomplishment was possible because of our mentor, teacher and friend Murray Goodman, who was always ready to encourage laboratories and scientists from all over the world to explore the potentialities of peptides. Our research group at Naples became part of the peptide community thanks to the stimulus and help constantly given by Murray. The path to research in peptide chemistry opened by Murray is now flourishing

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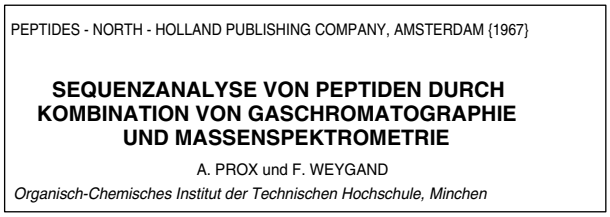
‡ Selected paper part of a special issue dedicated to the memory of Murray Goodman.

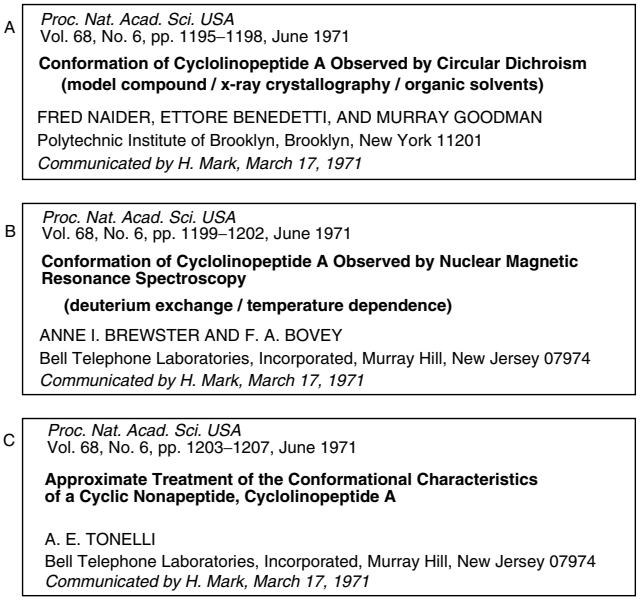

PEPTIDES - NORTH - HOLLAND PUBLISHING COMPANY, AMSTERDAM {1967}

SEQUENZANALYSE VON PEPTIDEN DURCH KOMBINATION VON GASCHROMATOGRAPHIE UND MASSENSPEKTROMETRIE

A. PROX und F. WEYGAND

Organisch-Chemisches Institut der Technischen Hochschule, Minchen

Figure 1 The 1967 communication by Prox and Weygand [2] on the chemical structure of CLA.


A

Proc. Nat. Acad. Sci. USA
Vol. 68, No. 6, pp. 1195–1198, June 1971

Conformation of Cyclolinopeptide A Observed by Circular Dichroism (model compound / x-ray crystallography / organic solvents)

FRED NAIDER, ETTORE BENEDETTI, AND MURRAY GOODMAN
Polytechnic Institute of Brooklyn, Brooklyn, New York 11201
Communicated by H. Mark, March 17, 1971

B

Proc. Nat. Acad. Sci. USA
Vol. 68, No. 6, pp. 1199–1202, June 1971

Conformation of Cyclolinopeptide A Observed by Nuclear Magnetic Resonance Spectroscopy

(deuterium exchange / temperature dependence)

ANNE I. BREWSTER AND F. A. BOVEY
Bell Telephone Laboratories, Incorporated, Murray Hill, New Jersey 07974
Communicated by H. Mark, March 17, 1971

C

Proc. Nat. Acad. Sci. USA
Vol. 68, No. 6, pp. 1203–1207, June 1971

Approximate Treatment of the Conformational Characteristics of a Cyclic Nonapeptide, Cyclolinopeptide A

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Communicated by H. Mark, March 17, 1971

Figure 2 (A) The 1971 paper by Naider *et al.* [3] on the CD of CLA. (B) The 1971 paper by Brewster and Bovey [4] on the NMR of CLA. (C) The 1971 paper by Tonelli [5] on calculations of the conformation of CLA.

through the scientific activities of his many alumni and scientists who have been in contact with him over the years as colleagues, but mainly as friends, all fascinated not only by his deep knowledge of peptide chemistry, but mainly by his warm personality.

BIOLOGY OF CLA

CLA as an Antamanide-like Inhibitor

Antamanide, cyclolinopeptide A and, to a minor extent, the peptide hormone somatostatin [8] all inhibit, to differing extents, the uptake into hepatocytes of bile salts as well as of phallotoxins, ethanol, cysteamine and dimethylsulfoxide. All these substances, as well as other cyclic peptides, are carried by the same mechanism through the hepatocyte membrane with which the cyclopeptide drugs form a strong competitive bond.

The identity of the membrane transport system for bile salts, phalloidin and antamanide was demonstrated by photoaffinity labelling [9]. Also, synthetic cyclopeptides derived from somatostatin [10] and CLA [11] were used to identify and isolate some components of the hepatic cholate transport system.

CLA as an Immunosuppressor

Recently, a cyclosporin-like immunosuppressive activity was attributed to CLA and AA [12]. In particular, CLA was found to possess immunosuppressive activity in a range comparable to that of cyclosporin A (CS-A) [13] with a mechanism for the immune activity which depends on the inhibition of interleukin-1 and interleukin-2 actions. This biological activity was assessed by the *in vitro* plaque forming cell test (PFC) and the *in vivo* delayed-type hypersensitivity test (DTH). However, the immunosuppressive activity of CLA was not confirmed in other tests. In fact, CLA does not show inhibition of T-lymphocyte activation, which modulates the immune response, as occurs with CS-A or the macrolide FK506 [14]. In fact, the immunosuppressive activity takes place through the interaction of CS-A or FK506 with two non-homologous cytoplasmic proteins (cyclophilin, CYP and FK506 binding protein, FKBP, respectively), endowed with peptidyl-prolyl *cis-trans* isomerase activity (called PPIase, or proline rotamase). The complex CYP-CS-A or FKBP-FK506, by *in vivo* specific binding with calcineurin (CN), a Ca^{2+}-calmodulin dependent serine/threonine phosphatase, forms a ternary complex, which inhibits an early step of the T-lymphocyte activation, thus modulating the immune response.

We recently reported evidence on the binding of CLA to bovine cyclophilin A. However, our preliminary results suggest that the peptide is not able to activate the immunosuppression mechanism. To clarify this point it would be valuable to determine whether the CLA-CYP complex is able to bind calcineurin.

STRUCTURE-ACTIVITY RELATIONSHIPS

The exhaustive research work carried out on AA, its analogues and related cyclopeptides of reduced ring size [7] allowed the conclusion that, despite the fact that no precise bioactive region was localized, any change of the two adjacent Phe residues in the Pro-Phe-Phe sequence resulted in a loss of bioactivity [15]. On the other hand, a parallelism was found between the bioactivity and the ability of the AA analogues to complex metal ions. Perhaps this property confers on the AA molecules a high tendency to adopt a suitable shape for binding either the substrate or the metal ion, thus achieving the bioactive conformation. Somatostatin, whose AA-like bioactivity is about 50–60 times lower when compared with the reference compound, shares with AA and CLA the presence of adjacent aromatic amino acids

Phe-Phe-Trp. This structural property was proven to be the essential requisite for bioactivity. The portion of the molecule not directly involved in the binding with the substrate seems to play a fundamental role in the modulation of bioactivity. On the basis of crystal-state and solution conformational analyses of synthetic cyclopeptides, a bioactive conformation was proposed for the Pro-Phe-Phe sequence.

Over the years we have prepared several homodetic and heterodetic cyclic CLA-analogues with different ring-size and amino acid composition. These compounds were tested for AA-activity, that is for their ability to inhibit the uptake into hepatocytes of bile salts, as well as phallotoxins, ethanol and other hepatotoxic substances. However, the activities shown by these analogues did not allowed a clear-cut assessment of the structure-activity relationships.

The Crystal-state Structure of CLA

The crystal-state conformation of CLA was independently reported by two research groups [16–19]. CLA was crystallized from several different solvent mixtures, leading to three different polymorphs: an orthorhombic form, grown from isopropanol–water, a monoclinic form, grown from N,N-dimethylformamide (DMF)–isopropanol, and a triclinic form, grown from acetone–benzene. In the unit cell, the orthorhombic, monoclinic and triclinic polymorphs contain no solvent, or DMF and water molecules, or a benzene molecule, respectively. Remarkably, in spite of the different environments in which the crystals were grown, the cyclolinopeptide A molecules in all crystal forms show exactly the same conformation (illustrated in the stereo diagram of Figure 3). The nonapeptide backbone has one *cis* peptide bond linking the two Pro residues (Pro^1-Pro^2). The backbone conformation of CLA is stabilized by the following intramolecular interactions:

(i) a $3 \rightarrow 1$ γ-turn stabilized by a H-bond between the N–H group of Leu^5 and the C=O group of Phe^3;

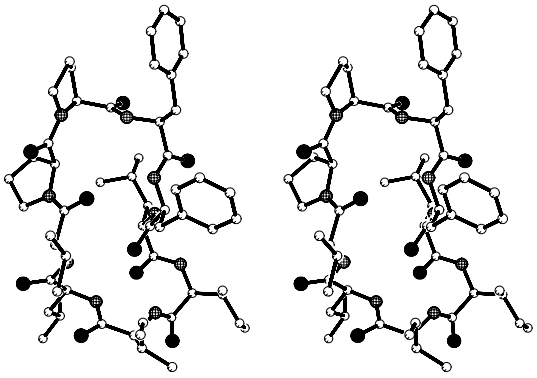

Figure 3 Stereo drawing of the conformation found in the crystal state for CLA.

(ii) two consecutive $4 \rightarrow 1$ β-turns stabilized by H-bonds occurring between the N–Hs of Ile^7 and Leu^8 and the C=Os of Phe^4 and Leu^5, respectively. These interactions are described as type-III and type–I β-turns, respectively;

(iii) an intramolecular $5 \rightarrow 1$ α-turn stabilized by a H-bond occurring between the NH group of Phe^4 and the C=O group of Val^9. Internally to this H-bonded structure, a *cis* peptide bond (between Pro^1 and Pro^2) is observed;

(iv) a $1 \rightarrow 5$ ring structure stabilized by a H-bond occurring between the N–H of Val^9 and the C=O of Phe^4. This turn can also be considered a C_{17}-ring structure if the atoms of the ring formed by the remaining portion of the cyclic molecule are considered.

Remarkably, L-Leu^8 presents backbone torsion angle values ($\varphi = 55^\circ$, $\psi = 48^\circ$) sufficiently high in energy, corresponding to the left-handed helical region of the conformational map and proper for a D-residue. However, in this conformation the residue is able to form two H-bonds involving its N–H and C=O groups with the C=O group of Leu^5 and the N–H group of Ile^6, respectively.

The conformational parameters of the Pro-Pro-Phe-Phe peptide sequence have a striking similarity to those of the homologous sequence of the natural cyclic decapeptide AA [20] with an average difference not greater than 22°. Furthermore, a similar stacking of the rings for the Pro^2-Phe^3 moiety is observed in both CLA and AA, and in various AA-analogue structures.

The conformation observed in the crystal state for the various forms of CLA clearly demonstrates that it represents a minimum energy conformation readily available to the cyclic nonapeptide under various crystallizing conditions. However, as shown in the next section, this conformation does not correspond to the bioactive conformation.

On the basis of the 3D-structural results found for CLA, an analogue was designed with a modified sequence by incorporating two Aib (α-aminoisobutyric acid) residues at positions 5 and 6 and a D-Ala (ala) residue at position 8 [21]. These changes in the sequence were expected to make the peptide backbone less flexible than that of CLA, tentatively freezing the conformation observed for CLA. The resulting nonapeptide c(Pro-Pro-Phe-Phe-Aib-Aib-Ile-ala-Val) (CLAIB), crystallized from two different solvent mixtures, gave two different, nearly isomorphous, hydrated forms containing either acetonitrile and two water molecules or two methanol and two water molecules, respectively.

In both structures the cyclic nonapeptide CLAIB assumes the same conformation which is also identical to that observed in the three different solvated crystalline forms of CLA. As expected, the substitution

of Leu[5], Ile[6] and Leu[8] with two conformationally constrained Aib residues and a D-Ala residue, respectively, does not perturb the backbone and the side-chain conformations, but actually increases the molecular rigidity, as demonstrated by a solution analysis (see next section). The intramolecular H-bond network in CLAIB is the same as that described in CLA. The backbones of the two molecules are practically superimposable.

Solution Conformation of CLA

A clear picture of the solution structure of CLA was given by a NMR study at low temperature (214 K) in an apolar solvent ($CDCl_3$) [16]. The flexibility of this cyclic nonapeptide is indeed comparable to that of linear peptides: the presence of two Pro residues, although effective in reducing the accessible conformational space, does not prevent the existence of a number of conformers that is larger than the number of experimental data in solution. This statement is particularly true for polar solutions. Only in the apolar solvent chloroform, at a very low temperature (214 K), did we succeed in preventing the formation of several different H-bonds between solvent molecules and the cyclic peptide.

CLA at room temperature exists as an equilibrium of many conformers, whilst at low temperature in appropriate apolar solvents it shows a single conformation essentially identical to that found in the crystal state [22–24].

Contrary to the results obtained in solution on CLA, CLAIB shows, even at room temperature, a unique conformation [21], which is identical to that found for CLA in the crystal state and in solution at low temperatures. The insertion of constrained residues effectively quenches the very high flexibility shown by CLA. The extent of rigidity of the CLAIB structure can be inferred by the similarity of the ROESY spectra at three different temperatures (270, 295 and 315 K). Its conformational rigidity could perhaps be one of the factors for the lower biological activity of CLAIB when compared with that of the native peptide. In fact, the peptide concentration required to inhibit the cholate uptake in hepatocytes by 50% (CD_{50}) increases from a value of 0.84 μM for CLA [23] to 30 μM for CLAIB. It seems likely that flexibility plays a role in the onset of the bioactive function of cyclic peptides of this class. This is not the first example of diminished activity in a peptide agonist as a consequence of the introduction of conformational constraints. In determining the bioactive conformation of peptides, the synthesis of conformationally restricted analogues has become a common practice. Successful examples are, *inter alia*, somatostatin [25,26] and enkephalin analogues [27,28]. Negative examples are the cyclic analogues of thymopentin [29,30], in which the conformational restriction introduced to reproduce the most probable conformation, as derived by NMR and energy minimization studies, led to a completely inactive analogue.

To prove the role of the single components of the supposedly active sequence Pro-Pro-Phe-Phe, each residue of this segment was replaced in turn with an L-Ala residue [31]. Although in none of the analogues was a dramatic decrease in bioactivity observed, a more evident effect occurred when the two adjacent Phe residues were involved, confirming the previous identification of their central role [31].

A preliminary NMR study [31] of the [Ala[2]] analogue in DMSO solution indicates a complex system of interconverting isomers, even if the substitution of one Pro residue with Ala eliminates the possible conformers related to the Xxx-Pro *cis-trans* isomerization. The lack of NOE effects and the clear evidence for the coexistence of different slow-exchanging isomers confirm a high degree of flexibility.

OTHER BIOLOGICAL ACTIVITIES FOR CLA?

The role played by CLA in linseeds remains to be determined. CLA, as well as other linear and cyclic peptides, present in good quantity in the seeds, must certainly have a role, given the use that popular medicine and ancient remedies have made and in some places still make of linseed compresses to reduce pain caused by traumatic and arthritic bone aches or skin inflammations. It is our belief that a thorough search for other possible biological activities of CLA could be rewarding. On the other hand, several peptides, which had been thought to possess only one well-defined bioactivity, were successively and serendipitously discovered to show other activities.

Our continuing efforts to understand the way in which CLA acts and to unravel its bioactive conformation, after half a century of studies on the 3D-structure of the native peptide as well as on those of many different analogues, are and will be our tribute to the memory of Murray Goodman who opened up this area of research with his enthusiastic encouragement and scientific support.

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