Metal Ion Modulation of Membrane Permeability Induced by a Polypeptide Template

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A correct folding into a well-defined 3-D structure represents the fundamental prerequisite for any protein to accomplish its biological function. Stabilization of such a structure occurs *via* hydrogen bond formation, hydrophobic and electrostatic interactions, or coordination to metal ions.[1] From the design of *de novo* proteins, we may expect to get insight into the process of their tertiary structure organization or to control the onset of specific functions.[2] Synthetic proteins have been obtained by connecting relatively small peptides of known secondary structure to a platform, as has been done by Mutter.[3] More recently, Ghadiri[4] has introduced a new strategy by assembling the peptide chains into the *de novo* protein *via* metal ion coordination. Modulation of the dynamics of designed proteins by metal ion coordination has been also reported.[5] Recently, these synthetic proteins have attracted considerable interest for the realization of artificial ion channels.[6−8]

The peptaibol family of antibiotics[9] constitutes a class of molecules that are thought to act through channel formation in cell membranes, leading to leakage of the cytoplasmic material and eventually to cell death. They are linear peptides containing a large portion of α-aminoisobutyric acid (Aib) residues, which strongly promote helix formation. Sequences of these peptides range from 19 amino acids (alamethicin)[10] to as low as 10 (trichogin)[11] or even six amino acids (trichodecenin).[12] In the case of alamethicin, the length of the helix is such as to allow complete spanning of the membrane bilayer, and channels are believed to form *via* the aggregation of several peptide monomers. For membrane activity, the shortest peptaibols require the presence of a long acyl chain at the N-terminus (hence, the term lipopeptides).[13] These helices, in particular those of trichogin, are able to span only half the bilayer, and consequently, membrane activity requires the alignment of two channels on the two lipids leaflets. The hydrocarbon moiety may allow binding of the peptide to the membrane with the correct geometry and alignment of the channels.

On these premises, we speculated that connecting two (or more) trichogin-like peptides *via* a proper spacer and controlling the tertiary structure of the resulting protein from extended (two consecutive peptide chains) to folded (a bundle of two or more peptide chains) could allow the control of permeability of a liposomal membrane. In the extended conformation, the length of this protein would be very similar to that of alamethicin, thus allowing complete spanning of the bilayer. By contrast, in the folded conformation, a cluster of short helical peptides would form which, lacking the hydrocarbon chain, should be unable to align to ensure formation of the active channel.

With this in mind, we connected to tris(2-aminoethyl)amine (TREN) three copies of the decapeptide[14] H-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (GLUGGLUGIL) (U = Aib) *via* a 4-carboxy-1-methylbenzene spacer to obtain the tris-decapeptide TREN derivative, **1**.[15] The spacer was introduced to avoid the involvement of the peptide in metal ion coordination with concomitant modification of its secondary structure. The decapeptide sequence is analogous to that of trichogin A IV. With respect to the natural lipopeptide sequence, there are two differences: (i) the Aib residue at the N-terminus has been omitted to simplify formation of the amide bond to the benzoyl derivative spacer and (ii) the C-terminal β-amino alcohol leucinol (Lol) has been replaced with the α-amino ester Leu-OMe. Both modifications are known to be uninfluential on the helical structure of the peptide.[11c,14,16] Furthermore, the Leu-OMe analogue of trichogin A IV (**3**) was shown to be as active as native trichogin A IV in affecting permeability of liposomal membranes.[17] For comparison, the tris-tripeptide derivative of TREN, **2,**[15] was also synthesized. It is known that coordination of N-functionalized TREN to Zn(II) involves, typically, formation of pentacoordinate complexes whose geometry is a trigonal bipyramid with the four amino groups and an extra ligand involved in the coordination.[18] Previous work from our laboratory[19] has shown that, upon binding of a Zn(II) ion, a benzyl-functionalized TREN derivative assumes a basket-like structure, with the three phenyl groups pointing in the same

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(1) (a) Jaenicke, R. Ed. *Protein Folding*; Elsevier: Amsterdam, 1980. (b) Creighton, T. E. *Proteins—Structure and Molecular Properties*; Freeman: New York, 1984. (c) Montelione, G. T.; Scheraga, H. A. *Acc. Chem. Res*. **1989**, *22*, 70.

(2) (a) Mutter, M.; Vuilleumier, S. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 535. (b) DeGrado, W. F.; Wasserman, Z. A.; Lear, J. D. *Science* **1989**, *243*, 622.

(3) Mutter, M.; Tuchscherer, G. G.; Miller, C.; Altmann, K.-H.; Carey, R. I.; Wyss, D. F.; Labhardt, A. M.; Rivier, J. E. *J. Am. Chem. Soc*. **1992**, *114*, 1463.

(4) (a) Ghadiri, M. R.; Fernholz, A. K. *J. Am. Chem. Soc*. **1990**, *112*, 9633. (b) Ghadiri, M. R.; Soares, C.; Choi, C. *J. Am. Chem. Soc*. **1992**, *114*, 825. (c) Ghadiri, M. R.; Soares, C.; Choi, C. *J. Am. Chem. Soc*. **1992**, *114*, 4000.

(5) Handel, T. M.; Williams, S. A.; DeGrado, W. F. *Science* **1993**, *261*, 879.

(6) (a) Åkerfeldt, K. S.; Lear, J. D.; Wasserman, Z. R.; Chung, L. A.; DeGrado, W. F. *Acc. Chem. Res*. **1993**, *26*, 191. (b) Stankovic, C. J.; Schreiber, S. L. *Chemtracts: Org. Chem*. **1991**, *4*, 1. (c) Sansom, M. S. P. *Prog. Biophys. Mol. Biol*. **1991**, *55*, 139. (d) Montal, M. *Curr. Opin. Struct. Biol*. **1995**, *5*, 501. (e) Åkerfeldt, K. S.; Kim, R. M.; Camac, D.; Groves, J. T.; Lear, J. D.; DeGrado, W. F. *J. Am. Chem. Soc.* **1992**, *114*, 9656. (f) Grove, A.; Mutter, M.; Rivier, J. E.; Montal, M. *J. Am. Chem. Soc*. **1993**, *115*, 5919. (g) Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1989**, *111*, 380.

(7) Stankovic, C. J.; Heinemann, S. H.; Delfino, J. M.; Sigworth, F. J.; Schreiber, S. L. *Science* **1989**, *244*, 813.

(8) (a) Oiki, S.; Danho, W.; Montal, M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2393. (b) Tosteson, M. T.; Auld, D. S.; Tosteson, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 707.

(9) Benedetti, E.; Bavoso, A.; Di Blasio, B.; Pavone, V.; Pedone, C.; Toniolo, C.; Bonora, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7951.

(10) (a) Pandey, R. C.; Cook, J. C., Jr.; Rinehart, K. L. *J. Am. Chem. Soc*. **1977**, *99*, 8469. (b) Fox, R. O.; Richards, F. M. *Nature* **1982**, *300*, 325.

(11) (a) Auvin-Guette, C.; Rebuffat, S.; Prigent, Y.; Bodo, B. *J. Am. Chem. Soc*. **1992**, *114*, 2170. (b) Gurunath, R.; Balaram, P. *Biopolymers* **1995**, *35*, 21. (c) Toniolo, C.; Peggion, C.; Crisma, M.; Formaggio, F.; Shui, X.; Eggleston, D. S. *Nature Struct. Biol*. **1994**, *1*, 908.

(12) (a) Fujita, T.; Wada, S.; Iida, A.; Nishimura, T.; Kanai, M.; Toyama, N. *Chem. Pharm. Bull*. **1994**, *42*, 489. (b) Monaco, V.; Formaggio, F.; Crisma, M.; Toniolo, C.; Shui, X.; Eggleston, D. S. *Biopolymers*, in press.

(13) Le Doan, T.; El Hajji, M.; Rebuffat, S.; Rajesvari, M. R.; Bodo, B. *Biochim. Biophys. Acta* **1986**, *858*, 1.

(14) Solution synthesis and characterization of the peptides discussed in this work are reported in the following: Toniolo, C.; Crisma, M.; Formaggio, F.; Peggion, C.; Monaco, V.; Goulard, C.; Rebuffat, S.; Bodo, B., in preparation.

(15) All new compounds have been fully characterized; synthetic procedures and analytical data are in the supporting information.

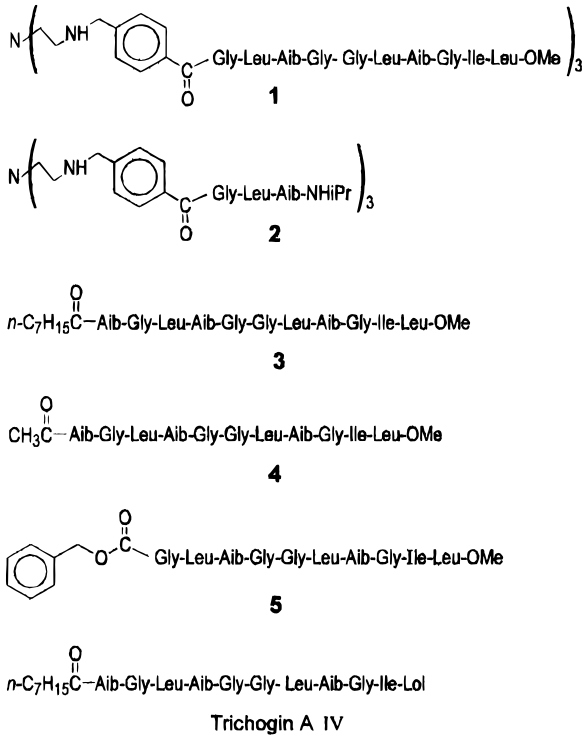
(16) Pirrone, L. Chemistry Degree Master Thesis, Department of Organic Chemistry, University of Padova, Padova, Italy, 1991.

(17) Bodo, B.; Rebuffat, S.; Goulard, C.; Peggion, C.; Monaco, V.; Formaggio, F.; Crisma, M.; Toniolo, C. In *Peptides 1994*; Maia, H. L. S., Ed.; ESCOM: Leiden, 1995; p 749.

(18) Prince, R. H. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Gillard, R. D., McCleverty, A., Eds.; Pergamon Press: London, 1987; pp 937−938.

0002-7863/96/1518-2505$12.00/0

direction. On the other hand, the uncomplexed ligand has no conformational constraints and, hence, has a quite flexible structure.



Modification of membrane permeability by compounds **1** and **2** was tested by monitoring trapped carboxyfluorescein (CF) leakage[13,20] from small unilamellar vesicles[21] composed of a 70:30 blend of fresh egg L-α-phosphatidylcholine (Sigma) and cholesterol. The percent of fluorescence increase was determined after 20 min of incubation at 25 °C and pH = 7.4 of CF-loaded vesicles in the presence of increasing concentrations of added peptide. The results are reported in Figure 1.

The tris-decapeptide derivative is quite effective in promoting CF leakage from the vesicles. However, when this molecule is added as the Zn(II) complex, the effect is greatly reduced. At a 4×10^{-7} M concentration, the uncomplexed polypeptide is almost 10 times more effective than the Zn(II) complex. Under identical conditions, very little leakage was induced by **2**, and no effect was observed with **4**, the acetyl homologue of **3**. Also, very little modification of permeability was observed with **5**, the N^{α}-carbobenzyloxylated peptide precursor in the synthesis of **1**. The permeation induced by **1** is not due to vesicle lysis, as dynamic light scattering experiments[22] performed with the vesicles in the presence or absence of **1** showed the same scattering intensity and size distribution. Furthermore, the observed effect is not due to a lower lipophilicity of the Zn(II) complex leading to a poorer binding to the vesicular membrane.[23] The rate of CF release in the presence of added **1** is almost 3 times faster than that observed under identical conditions for the trichogin-like peptide **3**. Clearly, putting together three peptide chains offsets the adverse effect induced by the absence of the hydrocarbon chain (as in the case of **4** or **5**).[24] The presence of Zn(II) controls the rate of the leakage in a reversible process. This is clearly shown in an experiment started by adding the **1**·Zn(II) complex to the CF-loaded vesicles: the rate of release[25] of CF can be increased by addition of a 25-fold excess of EDTA, which removes the metal ion from the complex (Figure 1, inset), transforming it into the more efficient polypeptide **1**. The EDTA complex is too hydrophilic to interact with the bilayer membrane and does not affect its permeability.

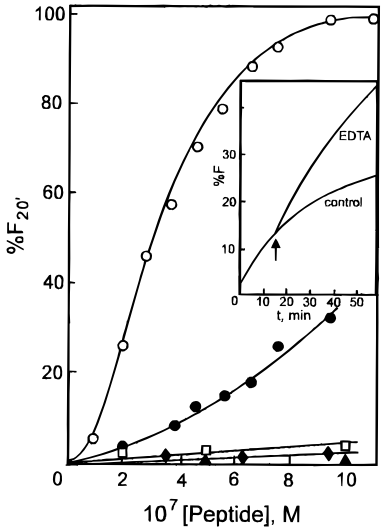


Figure 1. Relative amount of released CF after 20 min as a function of the concentration of added peptide. Conditions: [lipid] = 4×10^{-5} M, pH = 7.4 (0.01 M HEPES buffer), [NaCl] = 0.1 M; temperature, 25 °C. ○, **1**; ●, **1**·Zn(II); □, **2**; ▲, **4**; ♦, **5**. Inset: Time course of the increase of fluorescence upon addition of 5.6×10^{-7} M **1**·Zn(II); the arrow indicates when excess EDTA (1.5×10^{-5} M) was added to one of the two cuvettes while the other was left unchanged (control).

In conclusion, we succeeded in the synthesis of a polypeptide able to affect permeability of a model membrane and showed that the observed effect can be controlled by addition (or removal) of Zn(II) ions. Likely, this is due to a conformational change of the polypeptide in the membrane controlled by the formation of the metal complex. Molecular models indicate that the polypeptide is potentially able to span the bilayer (38 Å) in its fully stretched conformation (∼43 Å), while the folded Zn(II) complex is too short (∼24 Å). Work is in progress aimed at defining the minimal peptide length required to affect permeability and the mechanism operative in this system.

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Supporting Information Available: Syntheses and analytical data for compounds **1** and **2** (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(19) Scrimin, P.; Tecilla, P.; Tonellato, U.; Valle, G.; Veronese, A. *J. Chem. Soc., Chem. Commun.* **1995**, 1163.

(20) Winstein, J. N.; Yoshikami, S.; Henkari, P.; Blumenthal, R.; Hagins, W. H. *Science* **1977**, *195*, 489. λ_{exc} = 488 nm and λ_{em} = 520 nm. Experiments were started by addition of the peptide as a methanolic solution, and the maximum amount of solvent added was 1.5% (v/v). Control experiments showed that this amount of methanol did not affect vesicles permeability. 100% CF content was determined by complete vesicles disruption with excess Triton X100.

(21) Vesicles were prepared by sonication, and their size was analyzed by dynamic light scattering. Details are given in the supporting information.

(22) Nicomp 370 autocorrelator equipped with a Spectra-Physics 2016 argon laser operating at 488 nm. Note, however, that at high **1** (or **1**·Zn(II)) concentration (>8×10^{-7} M), a discrete population of unbound peptide starts to form, suggesting an equilibrium between membrane-bound and unbound peptide.

(23) This is highlighted by the behavior of **1** and **1**·Zn(II) in the aqueous medium used for the experiments. When [**1**] > 4.0×10^{-7} M and [**1**·Zn(II)] > 2.2×10^{-7} M, aggregates begin to form, as revealed by the increase of scattered light above these concentrations. These two concentrations may be taken as a rough estimate of the hydrophobicity of the two systems, which are very similar. This is hardly surprising, since **1** is triprotonated at pH = 7.4.

(24) Similar results have been obtained connecting together two undecapeptides with the same sequence of trichogin *via* a succinimidoyl spacer (see ref 14).

(25) CF release kinetics are not first order. This has been observed for alamethicin, too: Schwarz, G.; Robert, C. H. *Biophys. J.* **1990**, *58*, 577.