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The amphipathic α-helix concept

Application to the de novo design of ideally amphipathic Leu, Lys peptides with hemolytic activity higher than that of melittin

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Abstract

An original serie of 12- to 22-residue-long peptides was developed, they are only constituted by apolar Leu and charged Lys residues periodically located in the sequence in order to generate ideal highly amphipathic α-helices. By circular dichroism, the peptides are proven to be mainly α-helical in organic and aqueous solvents and in the presence of lipids. The peptides are highly hemolytic, their activity varies according to the peptide length. The 15-, 20-, and 22-residue-long-peptides have $LD_{50} \sim 5 \times 10^{-8}$ M for 10^7 erythrocytes, i.e. they are 5–10 times more active than melittin, and are indeed several orders of magnitude more active than magainin or mastoparan.

Key words: Amphipathic helix; Hemolysis; Cytotoxic peptide

1. Introduction

Cytotoxic amphipathic peptides represent a wide variety of natural compounds secreted by living cells; they are generally basic. Melittin (Mel), the best known with the highest hemolytic activity, comes from bee venom. It has a 26-amino acid residue-long sequence with a characteristic cluster of Lys and Arg residues at the C00-terminus which is required for activity [1,2]. δ-Hemolysin from *Staphylococcus aureus* behaves similarly although it is more polar and has no net charge at pH 7.5 [2,3]. Magainins, 23-residue-long peptides from frog skin, are essentially toxic for bacterial strains while being poorly hemolytic [2,4]. Bombolitins, mastoparans and related peptides are shorter, down to 14 residues. They are generally weakly hemolytic despite having cytotoxic properties [2]. Numerous studies have been performed to understand structure–function relationships; while only few have been devoted to rationalizing their sequences and to generating new more active peptides. In a pioneering work, De Grado et al. first simplified the Mel sequence creating an analog with an ideal amphipathic α-helical sequence from position 1 to 19 followed by the 20–26 natural segment [5]. This led to a more active peptide which proves that the kink in the α-helix is not required for hemolytic activity [5,6]. More recently, Mel hybrids with reversed sequence (16–26) (1–13) showed no hemolytic activity despite the fact that they remain antibacterial [7]. The synthesis of more than 50 analogs with systematic substitutions by Leu or single residue deletion proved that some residues play an essential role, e.g. Lys_7, Trp_{19}, and confirmed that a large α-helical amphipathicity is the main requirement for activity [8–10].

In this work we speculate that an ideally amphipathic α-helix with positively charged residues periodically located all along the peptide chain should have an activity similar to that of Mel. Such a de novo synthesis has already proved successful to mimic hormones or calmodulin-binding peptides [11]. In this work we associated the strongly apolar and helix-forming Leu residue to the basic one, Lys, which is the more frequent in the natural cytotoxins. Instead of using a 1:1 Leu-to-Lys ratio to generate highly polar and charged peptides, as recently studeid [12], we selected a 2:1 ratio. This allowed us to generate peptides of about the same size and charge as the natural toxins, more hydrophobic than those already studied in literature.

Here we document the hemolytic activity and the spectroscopic properties of a series of peptides whose lengths varied from 12 to 22 residues.

2. Materials and methods

2.1. Peptide synthesis

Seven peptides, 12–22-residues-long, were synthesized by SPM, using the base labile Fmoc-protecting group for the α-amino-terminus and

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Abbreviations: L_iK_j, peptide composed of i residues Leu and j residues Lys; Dns, dansyl; Mel, melittin; Lyso-PC, lysophosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; SUV, small unilamellar vesicles; Hα, α-helix.

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TFA-cleavable protecting groups for the various functionalities of the amino acid side chains. The chain assembly was performed on an automated peptide synthesizer (ABI, model 430A). The synthesis was developed by resin splitting, removing parts of the resin at respective positions and continuing with the rest. At the end of the syntheses the N_{term}Fmoc groups are removed and the resulting free amino groups are reacted with a ten-fold excess of dansyl chloride in NMP. After completion, the peptides were cleaved from the resin using a mixture of TFA/Anisol/water (90%:5%:5%). Peptides were purified by HPLC on a CN column (M&N) operated in the reverse phase mode, using a gradient of 35% to 100% CH_3CN. The single peak eluting peptides have retention times determined on an analytical CN column (M&N, ET2508/4 Nucleosil 300-7 CN), which increase from 21 to 24.4 and 27.7 min for the 12-, 15- and 22-residue-long peptides. The peptides' compositions were checked on an amino acid analyzer (ABI, model 420A) and their sequences were confirmed on an ABI model 477A before reacting the peptides with dansyl chloride. The mass (Finnigan MAT, model TSQ700/ESI) of the 12-mer is in good agreement with the theoretical one (MW_{exp} = 1669.0 $g \cdot mol^{-1}$ vs. MW_{theor} = 1669.9 $g \cdot mol^{-1}$). The purity of all peptides is > 95% based on HPLC, they are all soluble in MeOH and their concentrations were estimated using the molar extinction coefficient of the dansyl group, $\varepsilon_{320\ nm} = 4640\ M^{-1} \cdot cm^{-1}$.

2.2. Hemolytic assay

Fresh human blood from a healthy O^+ donor was collected on citrate and centrifuged 3 min at 980 × *g*. The erythrocyte pellet was washed several times and finally resuspended in 20 mM Tris, 130 mM NaCl buffer, pH 7.5. For each assay, peptides were diluted at the defined concentration in 1 ml of buffer, then 0.5 ml of the stock solution of diluted cells were added corresponding to 10^7 cells/assay in all experiments. The mixture is incubated at 37°C for 2 h and the % of hemolysis is defined by measuring the absorbance at 414 nm of the supernatant after pelleting ghosts and undamaged cells for 3 min at 1000 × *g*. The 0% hemolysis is obtained incubating erythrocytes with isotonic buffer alone while the 100% hemolysis is found in pure water. The data reported are generally a mean of two or three independent measurements.

2.3. Spectroscopic measurements

Absorption spectra were routinely recorded with a Pye Unicam Philips spectrometer 8800. Far UV dichroic absorption spectra were recorded on an AVIV 62DS spectrometer in thermostated 0.1 or 1 cm cuvettes, respectively, for low and high peptide concentrations. The reported data are the mean of at least 3 independent measurements. Backgrounds were always substracted.

2.4. Materials

The phospholipids used were from Lipid Products (Nutfield, UK) and Avanti Polar Lipids (Birmingham, USA). The lyophylized powder is hydrated in Tris 20 mM, pH 7.5, buffer, homogeneized on a vortex and bath sonicated for 2 min until obtaining homogeneously scattering solutions at 5 mg/ml which mainly consist of SUVs. Melittin was purchased from Serva, synthetic magainin II from Neosystem (France) and poliste mastoparan from Peninsula Laboratories (USA).

3. Results and discussion

3.1. Peptide design and amphipaticity

The primary structure of the peptides synthesized are summarized in Table 1. The 22-residue-long peptide constitutes the parent sequence, the shorter ones correspond to increased shortenings from the N_{term} side. The N-terminus is blocked by the dansyl group.

The design of these sequences used minimal requirements to generate highly amphipathic peptides when folded in α-helix. The roughly 2:1 molar ratio of Leu-to-Lys allows peptides in the range of 20-mers to be obtained. These peptides have a net charge and an hydrophobic sector not very different from that of Mel [2] despite the fact that there is *no* relation at all in the sequences or in the charge topology.

When folded in an α-helix, such sequences generate highly amphipathic peptides. This is shown by the Edmundson wheels for the shortest and longest peptides (Fig. 1). All the peptides fold in such a way that all Lys are confined within a sector of about a 100° width regardless of chain length. This corresponds to what occurs in cytotoxins [14]. Using the Eisenberg's hydrophobic scale and moment method [13], these L_iK_j peptides have an hydrophobic moment per residue $<\mu_H> \simeq 0.43 \pm 0.03$. Such a value is quite higher than those calculated not only for the L_iK_j serie previously described by Blondelle [12], $<\mu_H> \simeq 0.29$, but also for 1–21 Mel, $<\mu_H> \simeq 0.28$, and even for δ-toxin, $<\mu_H> \simeq 0.38$. Indeed the total hydrophobic moment of the peptides, μ_H, increases linearly with their lengths from $\mu_H \simeq 5.1$ for the shorter 12-mer up to $\mu_H \simeq 9.8$ for the larger one. Such values are larger than that estimated for the 1–21 fragment of Mel, $\mu_H \simeq 6$, and compare to that of the 26-residue-long amphipathic rod of δ-toxin $\mu_H = 9.87$.

3.2. Secondary structure of the peptides

In buffer or methanol solutions the far UV dichroic spectra show that the longer the peptide the higher the absolute values for their dichroic absorbance at extrema

Table 1
Definition and sequences of the studied peptides

Peptide length	Composition	Sequences (positions 1, 4, 8, 11, 15, 19, 22)
22	$L_{15}K_7$	Dns K L L K L L L K L L K L L L K L L L K L L K
21	$L_{15}K_6$	Dns L L K L L L K L L K L L L K L L L K L L K
20	$L_{14}K_6$	Dns L K L L L K L L K L L L K L L L K L L K
19	$L_{13}K_6$	Dns K L L L K L L K L L L K L L L K L L K
18	$L_{13}K_5$	Dns L L L K L L K L L L K L L L K L L K
15	$L_{10}K_5$	Dns K L L K L L L K L L L K L L K
12	L_8K_4	Dns K L L L K L L L K L L K

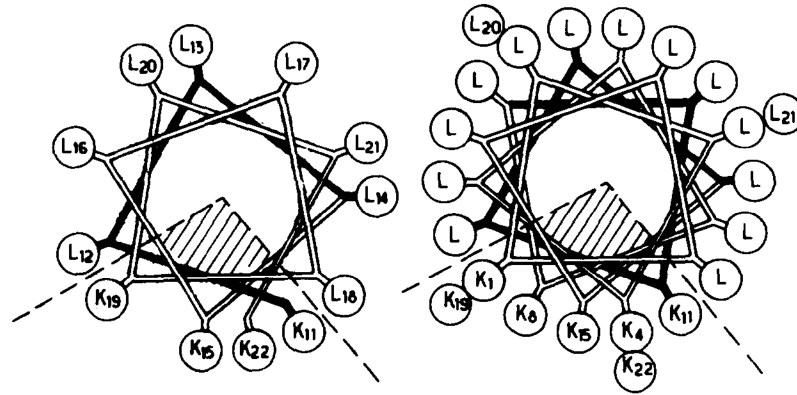

Fig. 1. Helical wheel representation of model peptides. Left: L_8K_4; right: $L_{15}K_7$, for a better comparison the amino acid numeration is the one used for the longest peptide.

(Fig. 2). The spectra are characteristic of what is expected for mainly α-helical structures (Fig. 2). The characteristic value at 222 nm decreases progressively, from $\theta_{222} \simeq -14{,}800$ deg·cm^2·dmol^{-1} for L_8K_4, down to $\theta_{222} = -35{,}000$ deg·cm^2·dmol^{-1} for the 22-residue-long peptide. For this last compound, the whole spectrum from 190 to 340 nm, can also be fitted with that of a pure α-helical structure.

When Lyso PC or DMPC vesicles are added to the peptides in buffer solution at 30°C, significant changes in the CD spectra are observed. At high lipid-to-peptide molar ratios (≥ 100), characteristic absolute values of θ_{195} and θ_{222} are increased, as seen in Fig. 3a,b, indicating an increase in the α-helix content. In DMPC only the shorter L_8K_4 has a low α-helix content (~40%), $L_{10}K_5$ like $L_{13}K_6$ are about 80% α-helix, the longest $L_{15}K_7$ accounting for 100% α-helix.

Direct use of CD proteins data to fit spectra or analyze θ_{222} values of short peptides in terms of α-helix content can be questioned since the characteristic dichroic values of each type of structure depend on the number of the residues involved [15]. In Fig. 3 the data from L_iK_j peptides compare quite well to those of highly favoured α-helical peptides [16]. Moreover, the concomitant changes of θ_{195} and θ_{222} values when the L_iK_j peptide length increases are coherent to what was expected from theoretical and statistical calculations [15,17]. Such general agreement allows us to conclude that the longer peptides when bound to lipids behave essentially as pure α-helices while the shorter ones, 12- and 15-mers, adopt significantly the same α-helical structure. A CD study versus temperature (data not shown) strengthens such conclusions that the longer the peptides the more stable their structure.

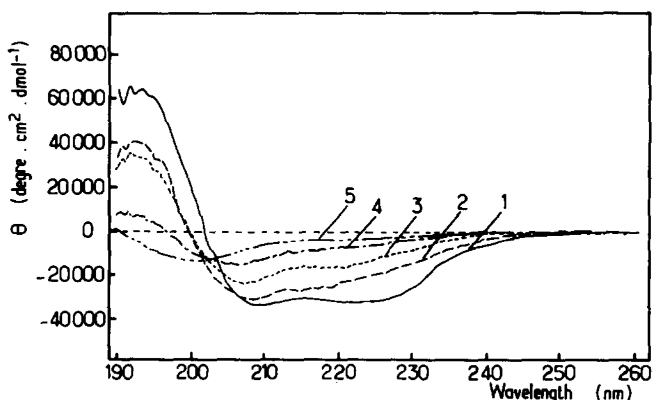

Fig. 2. CD spectra of the studied peptides in aqueous solution (Tris 1 mM, pH = 7.5) peptide concentration: 5 μM. 1 = DNS $L_{15}K_7$; 2 = DNS $L_{13}K_6$; 3 = DNS $L_{10}K_5$; 4 = DNS L_8K_4; 5 = Mel.

3.3. *Hemolytic activity of the L_iK_j peptides*

The synthesized peptides were assayed for hemolysis of human red blood cells and dose–response curves are shown on Fig. 4. The 20- and 22-mer peptides display an activity in the nanomolar range with an LD_{50} value ~4–6 × 10^{-8} M. In identical experimental conditions Mel used as a reference has an $LD_{50} \simeq 0.4$–0.5 μM, i.e. the peptides synthesized herein are about 6–10 times more active. Shortening the peptides decreases the hemolytic activity: the 19-mers has an $LD_{50} \simeq 2.1$ μM and the 18-mers LD_{50} increases up to 4.7 μM, i.e. about 80 times larger than that of the 22-mers. However, decreasing the peptide length further, a high activity is found again for the $L_{10}K_5$, $LD_{50} \simeq 0.1$ μM, while the shorter L_8K_4 peptide remains as active as the 18-mers. Even though the Dns group favors the activity, the 22-mer $L_{17}K_5$ with the free N-terminus remains three times more active than Mel, $LD_{50} = 0.16$ μM (data not shown).

The hemolytic activities found herein are indeed much larger compared to those of the naturally occurring cytolytic peptides. This is true for Mel (Fig. 4), and it is even much more drastic when compared to the barely active magainin, and the shorter mastoparan which only gave significant hemolysis (≥ 10%) at high concentration (≥ 10 μM).

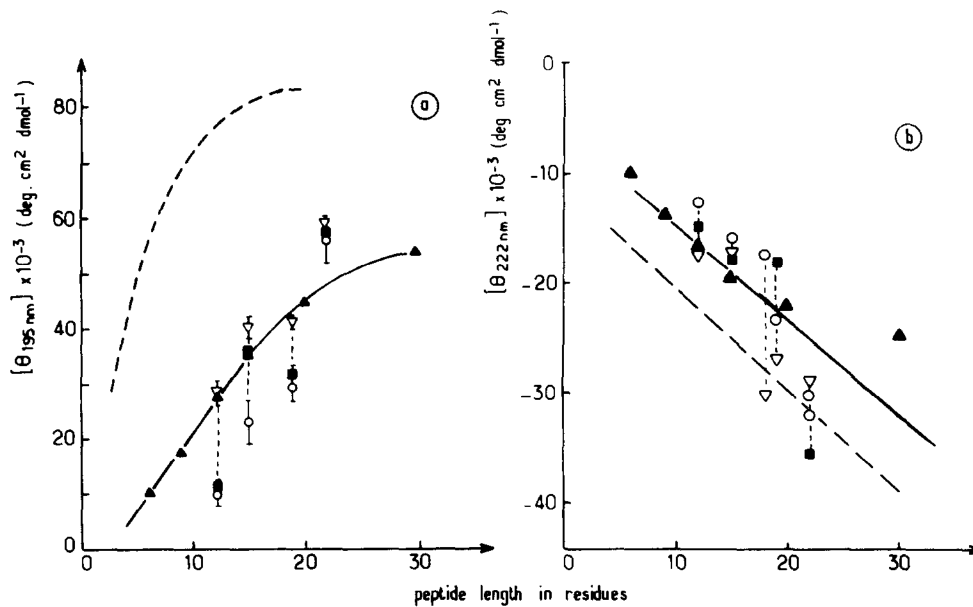

Fig. 3. Changes in the molar ellipticity per residue at 222 nm (a) and 195 nm (b) versus the peptidic chain length in different conditions. (○) peptides in buffer at 5 μM; (■) peptides in MeOH at 50 μM; (▽) peptides bound to lysolecithin micelles, PL/pept = 100; [Peptide] = 5 μM; (---) calculated laws by Chen et al. [15]; (–) and Katakai et al. [16]; (▲) data from Manning et al. [17] for $(MML)_n$ peptides in apolar medium.

4. Discussion and conclusion

The extreme reductionism to generate amphipathic peptides with only Leu and Lys residues, periodically located along the sequence, leads to compounds which are proven to be an α-helix, particularly when bound to lipids. Peptides have a very highly hemolytic activity, up to 5–10 times that of Mel for 15-, 20-, 21- and 22-residue-long peptides.

Therefore, a highly sophisticated sequence with numerous different, more or less polar, residues is not required to get hemolytic activity. The unique concept of generating an ideally amphipathic α-helical rod with apolar and charged residues properly located periodically at each turn is sufficient to fulfill the requirements to get the strongest hemolytic peptides.

Two recent attempts to get hemolytic model peptides were developed. First, Blondelle and Houghton [12] synthesized peptides with a Leu/Lys ratio = 1 and Tytler et al. [18] proposed an 18-residue-long peptide to be the archetype for lytic peptides. However, in both studies authors did not provide structural data: they only measured a high amount of lysis ($\simeq$ 80%) at two peptide concentrations (43 μM and 3 μM). Since they used about a ten-fold higher cell concentration compared to that used in our experiments, one can roughly estimate the 18-L-consensus peptide [18] has an hemolytic activity about three-fold higher than the one of melittin, close to that of our 22-mer with free N-terminus. However, the activities of L_iK_i peptides are 4–7 times lower than that of Mel, e.g. about 50 times less active compared to the most active peptides studied herein.

The apolar cluster constituted by 8 Leu residues like in the 12-mer allow the high partitioning into lipids required for activity. The real need for highly charged

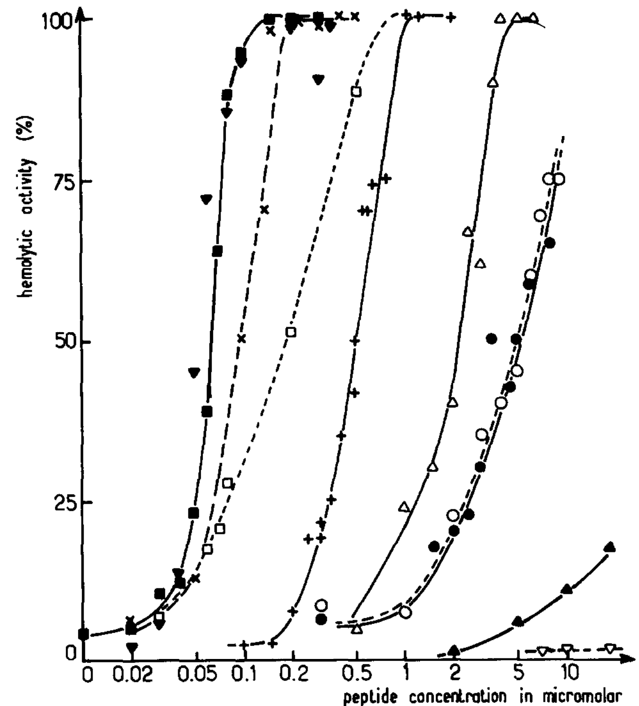

Fig. 4. Hemolysis induced by the amphipathic peptides on 10^7 cells/ml at 37°C, Tris buffer 20 mM, 130 mM NaCl, pH 7.5. Natural cytotoxins: (+) Mel; (▲) Magainin; (▽) Mastoparan. Synthetic dansylated peptides of increasing length: (○) 12-; (×) 15-; (●) 18-; (△) 19-; (▼) 20-; (□) 21-; (■) 22-mers.

peptides has not been properly understood until now. Charges allow peptide solubility and prevent a massive aggregation in water. Moreover, they can play a role in binding to membranes. The charge topology herein totally differs from that of Mel but is rather similar to that of other amphipathic peptides remaining more probably flat on the bilayer [19,20].

Taking into account the amphipathic concept, a monotonous increase in activity was expected versus the peptide length, a threshold being anticipated from the study of δ-toxin analogues [3]. This is not what is observed since a maximum of the activity of 15-residue-long peptide is followed by a drop of the hemolytic activity for 18- and 19-residue-long-ones. This dual behavior could be related to the self-association of peptides in solution, which should increase in parallel with the peptide length and would decrease peptide activity.

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References

[1] Habermann, E. (1980) in: Natural Toxins (Eaker D. and Waström, T. eds.) Pergamon Press, New York, pp. 173–181.
[2] Cornut, I., Thiaudière, E. and Dufourcq, J. (1993) chap. 8 'The amphipathic helix in cytotoxic peptides', in: The Amphipathic Helix (Epand R.M. Ed.), CRC Press, Boca Raton, pp. 173–219.
[3] Thiaudière, E., Siffert, O., Talbot, J.C., Bolard, J., Alouf, J.E. and Dufourcq, J. (1991) Eur. J. Biochem. 195, 203–213.
[4] Bevins, Ch.L. and Zasloff, M. (1990) Annu. Rev. Biochem. 59, 395–414.
[5] De Grado, W.F., Kezdy, F.J. and Kaiser, E.T. (1981) J. Am. Chem. Soc. 103, 679–681.
[6] Dempsey, C.E., Bazzo, R., Harvey, T.S., Sypereck, I., Boheim, G. and Campbell, I.D. (1991) FEBS Lett. 281, 240–244.
[7] Boman, H.G., Wade, D., Boman, I.A., Wählin, B. and Merrifield, R.B. (1989) FEBS Lett. 259, 103–106.
[8] Blondelle, S. and Houghten, R.A. (1991) Peptide Res. 4, 12–18.
[9] Blondelle, S. and Houghten, R.A. (1991) Biochemistry 30, 4671–4678.
[10] Blondelle, S., Simpkins, L.R., Pérez-Payá, E. and Houghten, R.A. (1993) Biochim. Biophys. Acta 1202, 331–336.
[11] O'Neil, K.T. and de Grado, W.F. (1990) Trends Biochem. Sci. 15, 59–64.
[12] Blondelle, S. and Houghten, R.A. (1992) Biochemistry 31, 12688–12694.
[13] Eisenberg, D., Weiss, R.M. and Terwilliger, T.C. (1982) Nature 299, 371–374.
[14] Segrest, J.P., de Loof, H., Dohlman, J.G., Brouillette, C.G. and Anantharamaiah, G.M. (1990) Proteins 8, 103–117.
[15] Chen, Y.H., Yang, J.T. and Chan, K.H. (1974) Biochemistry 16, 3350–3359.
[16] Katakai, R., Wanikawa, K. and Saga, K. (1990) Biopolymers 30, 815–819.
[17] Manning, M.C. and Woody, R.W. (1991) Biopolymers 31, 569–586.
[18] Tytler, E.W., Segrest, J.P., Epand, R.M., Nie, S.-Q., Epand, R.F., Mishra, V.K., Venkatachalapathi, Y.V. and Anantharamaiah, G.M. (1993) J. Biol. Chem. 268, 22112–22118.
[19] Chung, L.A., Lear, J.D. and De Grado, W.F. (1992) Biochemistry 31, 6608–6616.
[20] Reynaud, J.A., Grivet, J.P., Sye, D. and Trudelle, Y. (1993) Biochemistry 32, 4997–5008.