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Selective labelling of melittin with a fluorescent dansylcadaverine probe using guinea-pig liver transglutaminase

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Melittin, a C-terminal glutamine peptide, incorporated the fluorescent probe monodansylcadaverine (DNC) when catalysed by guinea-pig liver transglutaminase and Ca^{2+}, as determined by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). A 1:1 adduct DNC-melittin was identified in which a single glutamine residue out of two, i.e. Gln_{25}, acts as acyl donor. Incubation of melittin with transglutaminase in the absence of DNC originated high molecular mass complexes indicative that the peptide lysine residue can act as an acyl acceptor. The DNC-melittin was about 3 times more active in the lysis of red cell membranes than native melittin. Fluorescence study of the labelled melittin in the submicromolar range where it is active on cells showed that while totally exposed to solvent in methanol solution, both Trp and dansyl groups are buried in buffer solution. This strongly suggests that DNC-melittin is self-associated and indeed more active than the native melittin in the same conditions.

Melittin; Transglutaminase; Dansyl-labelling; HPLC

1. INTRODUCTION

Melittin is a basic and amphiphilic peptide of 26 amino acids, Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-CO-NH_2, that constitutes about 50% of the dry weight of bee venom [1]. Melittin binds to model and cell membranes, causing their lysis through as yet not completely understood mechanisms. At present, both the aggregation state and the orientation of the amphipathic α-helix with respect to the membrane remain controversial [2-4].

To obtain a better understanding of the mechanism of membrane lysis induced by such toxins, the microscopic localization of these effectors in the cell membranes at physiological concentrations is needed. In relation to this, efforts have been made in the last few years to chemically modify melittin with fluorescent or spin-label probes [5-9]. The labelled peptides should exhibit a high emission in the visible, which allows differentiation from other cell proteins and to detect it in the subnanomolar range. A clear disadvantage of these methods is that the chemical procedure is a laborious, long-lasting task, and it is generally very difficult to obtain experimental conditions suitable for the selective modification of a single residue.

Recently, the usefulness of the transglutaminase reaction has been recognised for in vitro selective modifications of small peptides and proteins with primary amines [10-12]. In particular, fluorescent amine probes have been used in structural studies [12,13]. Transglutaminases are Ca^{2+}-dependent enzymes catalysing an acyl-transfer reaction between γ-carboxamide groups of peptidic Gln residues and primary amines or ϵ-groups of peptide-bound Lys residues [14]. In this paper, we show that melittin, a C-terminal glutamine peptide with two adjacent glutamine residues (Gln_{25} and Gln_{26}) can be selectively labelled in the first one with guinea-pig liver transglutaminase and DNC. It results a more active peptide which looks to be much more stable as self-associated compared to the native peptide.

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Abbreviations: DNC, dansylcadaverine; DNC-Mel, dansylcadaverine-melittin; DNS-Gly, dansyl-glycine; EDTA, ethylene-diaminetetraacetic acid; HPLC, high-performance liquid chromatography; Mel, melittin; TGase, transglutaminase EC 2.3.2.13; TLC, thin-layer chromatography; UV, ultraviolet

2. EXPERIMENTAL

2.1. *Materials and chemicals*

Melittin and monodansylcadaverine, *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalene sulphonamide (DNC) were from Serva (Heidelberg, Germany). Guinea pig liver transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) was from Sigma (St. Louis, MO). Salts, buffers and reagents were of the highest purity available.

2.2. *Assay for transglutaminase activity*

The reaction was carried out in 0.1 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM calcium chloride, 20 mM dithiothreitol, 2 μM of TGase and the desired amounts of melittin and DNC. Reaction mixtures were incubated at different times at 37°C and stopped by heating at 60°C for 5 min [15].

2.3. *Chromatographic measurements*

For TLC analysis, 8 μl of reaction mixture were applied and dried on 0.2 mm layer thickness, silica gel 60, TLC plastic sheets from Merck (Darmstadt, Germany). Plates were eluted using a $CHCl_3/CH_3OH/NH_4OH$ mixture (2:2:1, v/v/v) and reaction products were visualized either by ninhydrin spraying or under UV-illumination ($\lambda = 254$ or 360 nm).

HPLC was performed with a Waters chromatograph (Waters Assoc., Mildford, MA), equipped with a F-1050 fluorescence spectrophotometer (Merck, Darmstadt, Germany). The column, an Ultrahydrogel 120, was eluted isocratically with 100 mM sodium acetate buffer, pH 4.4, containing 20% acetonitrile at room temperature. The fluorescence of the effluent was monitored with excitation at 320 nm and emission at 520 nm. When incubating with the enzyme, samples were centrifuged at 10000 rpm for 10 min before injection.

3. RESULTS

Incubation of monomeric melittin in the presence of TGase and DNC produced mainly a labelled molecular species visible under UV light after separation on silica gel plates with the chromatographic solvent system. The time course of DNC incorporation and the effect of three different melittin concentrations on the reaction are shown, as an example, in the TLC pattern in Fig. 1.

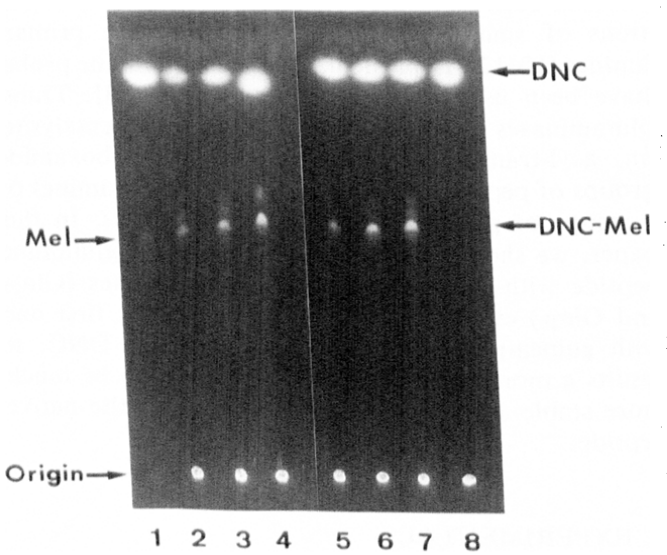

Fig. 1. Effect of melittin concentration and kinetics of the DNC-melittin modification by TGase. Melittin (20 μg, lane 2; 40 μg, lane 3; 100 μg, lane 4) was incubated at 37°C for 2 h with 2 μM TGase and 0.7 mM DNC (final volume, 100 μl). For time-course studies, 100 μg of melittin was incubated under the above conditions for 15 (lane 5), 60 (lane 6), and 120 min (lane 7). Blanks (lanes 1 and 8) were run in the absence of $CaCl_2$ or peptide, respectively. 8 μl aliquots were applied except for lane 1 (12 μl). Fluorescent bands were photographed under UV illumination.

These experiments indicate that melittin incorporates DNC, the covalent adduct having an $R_f = 0.63$ higher than native melittin, $R_f = 0.56$. The spot observed at the origin can be due in part to a non-specific binding between the enzyme and the fluorescent probe, as revealed from the blanks incubated in the absence of the acyl donor melittin (Fig. 1, lane 8). On the other hand, it is also very likely that some amount of high molecular mass complexes of labelled melittin can exist. This is supported by the fact that such complexes were detected when melittin was incubated with TGase in the absence of DNC, the external acyl acceptor amine. Thus, melittin acts as an intrinsic acyl acceptor through the involvement of lysine residues in the formation of inter- and/or intramolecular crosslinks with two potential glutamine residues (i.e. Gln_{25} or Gln_{26}), the most likely candidate being Lys_7 on the basis of its pK-value [16].

Although TLC is a sensitive, simple and direct method to follow mellitin modification (see Fig. 1), which allows detection of fluorescent adducts in the range of picomoles, it is time-consuming (about 3.5 h for a complete elution of TLC plates). Consequently, an HPLC-method has been developed in order to both directly visualize the time-course of the reaction [17], and completely separate modified and non-modified melittin as well as free DNC on the basis of differences in their molecular masses and hydrophobicities. Typical elution profiles for the transglutaminase assay with 20 mM Ca^{2+} or withhout Ca^{2+} are shown on Fig. 2. In the absence of Ca^{2+}, activator of the enzyme [14], two peaks are detected in UV at 7.6 and 30.7 ml, correspon-

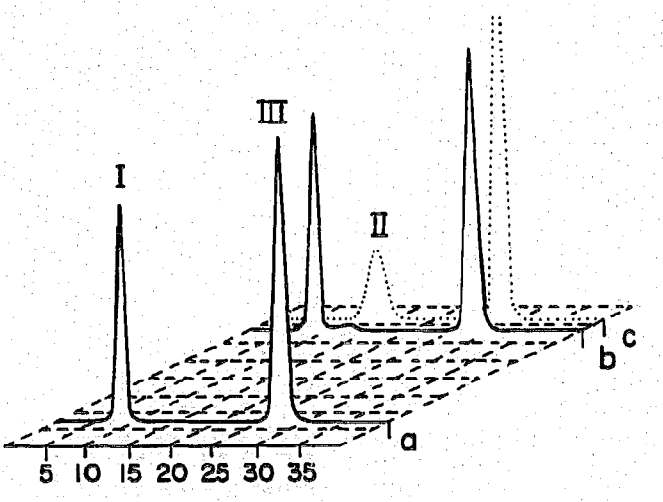

Fig. 2. Elution profiles for DNC-melittin modification by TGase detected by absorbance at 283 nm (——) or fluorescence (···). Melittin (100 μg) was incubated with TGase (2 μM, 37°C, 1 h, see legend of Fig. 1) without $CaCl_2$ (a) or in the presence of 20 mM $CaCl_2$ (b,c). Flow-rate 1.0 ml/min; injection volume, 10 μl. For clarity, the peak corresponding to dithiothreitol monitored by UV at 21 ml has been omitted.

ding to melittin (peak I) and free DNC (peak III) respectively (Fig. 2a), while only free DNC was detected at 30.7 ml monitoring the effluent fluorescence at 520 nm. Melittin-bound DNC was eluted at 12.8 ml (peak II) as shown in Fig. 2 (b and c). Since TGase catalyses the incorporation of DNC into melittin, peak II area increases while peak I and III areas concomitantly decrease from the initial values obtained in the Ca^{2+}-free condition.

The change in the melittin-bound DNC UV absorption is remarkable with respect to native peptide. A 1:1 stoichiometry was inferred for the DNC-melittin adduct using an extinction coefficient of 3860 $M^{-1} \cdot cm^{-1}$ at 332 nm for the dansyl chromophor. This species originated a single spot with $R_f = 0.63$ when controlled by TLC (see section 2).

Following digestion of DNC-mellitin by α-chymotrypsin, only one DNC-labelled peptide was eluted by reverse-phase chromatography using Sep-Pak C_{18} cartridges as described by Porta et al. [10] (results not shown). Amino acid analysis of this peptide after total acid hydrolysis indicated that it corresponds to the

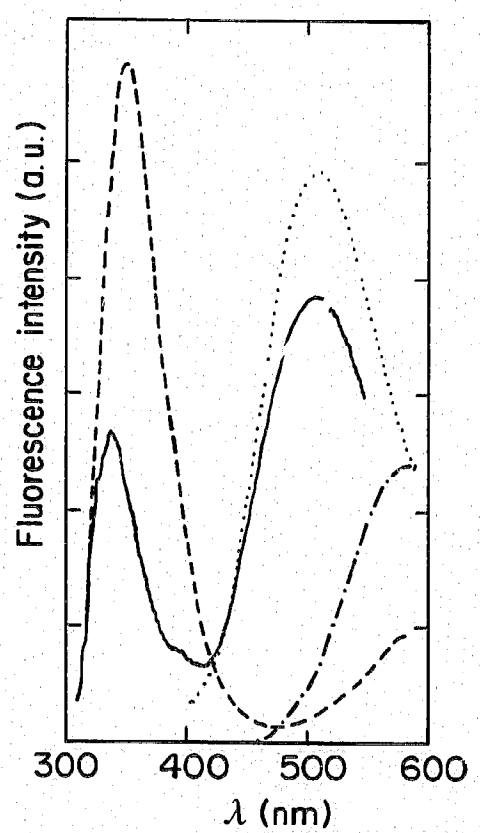

Fig. 3. Comparison of fluorescence emission spectra of DNC-melittin and the 1:1 mixture of native melittin and DNS-Gly. Corrected fluorescence spectra were obtained on a SLM 8000 spectrofluorimeter (Urbana, IL, USA) with 8 nm slit-width. DNC-melittin (——), excitation wavelength 280 nm; (···) excitation wavelength, 350 nm. 1:1 mixture (– –), $\lambda_{exc} = 280$ nm; (—●—), $\lambda_{exc} = 350$ nm. [peptide] = 0.17 μM; 20 mM Tris-HCl, 1 mM EDTA, pH 7.3, $T = 25°C$.

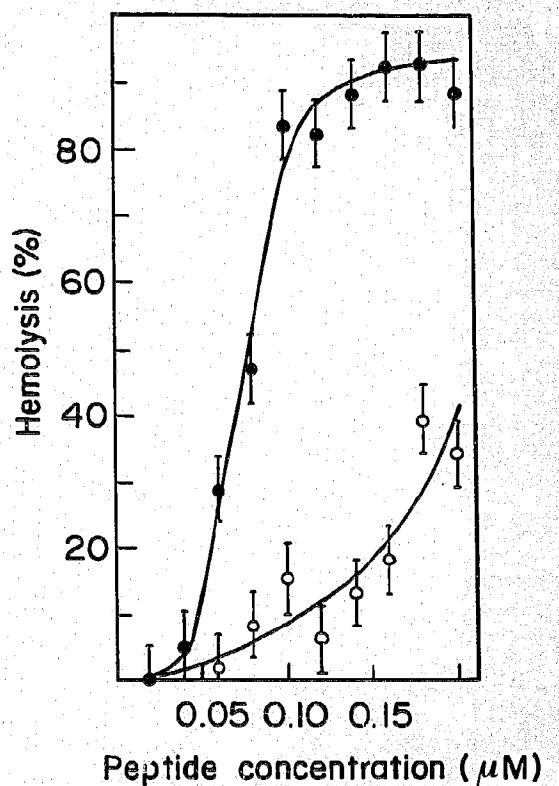

Fig. 4. Dose/response hemolysis of human erythrocytes induced by native (○) and DNC-melittin (●). Increasing amounts of melittin and DNC-melittin were added to washed erythrocytes suspension (3.5×10^6 cells/ml in Tris-HCl 20 mM, EDTA 1 mM, NaCl 130 mM, pH 7.3). The mixture was incubated for 1 h at 37°C and centrifuged at $800 \times g$ for 3 min. Hemoglobin in the supernatant was estimated spectrophotometrically at 541 nm. The total release was determined from hypotonic lysis in pure water.

20–26 C-terminal fragment. In addition, exhaustive digestion of labelled melittin with proteolytic enzymes (proteinase K, arginyl endopeptidase, carboxypeptidase A and B) using a similar procedure to that described by Cariello et al. for DNC-*N,N'*-dimethylcasein [18], followed by analysis of the released fragments by reversed-phase HPLC, indicates that only Gln_{25} acts as acyl donor in the TGase-mediated reaction. This is not surprising in the light of the specificity exhibited by TGase for peptide substrates with two consecutive glutamine residues [10,19].

When DNC-melittin fluorescence emission is monitored in methanol solution, on excitation at 280 nm one observes the emission of Trp at 340 nm and a weaker band at 540 nm due to the dansyl group. Both emission maxima are at the wavelength position of native melittin and DNS-Gly in that solvent. In contrast, as seen on Fig. 3, when DNC-melittin is dissolved in buffer at low concentration, its two emission bands differ compared to parent molecules, melittin and DNS-Gly. The emission maximum at $\lambda_{max} \simeq 330$–340 nm corresponds to a buried Trp residue compared to that of native

monomeric peptide in the same conditions (λ_{max} = 350 nm) [20]. The emission maximum at 505 nm corresponds to a totally buried dansyl group; under the same conditions, that of DNS-Gly is λ_{max} = 580–590 nm. Moreover, as seen on Fig. 3, the Trp fluorescence is severely quenched compared to that of native melittin whereas that of DNS is very significantly increased. The fluorescence intensity ratio of DNC for excitation wavelengths at 350 nm and 280 nm, is lower for DNC-melittin than for the mixture. When observation is settled at 505 nm, excitation spectra (not shown) bear a shoulder at 280 nm which arises from Trp. Then we can propose that resonance energy transfer occurs between different peptide chains from Trp to the dansyl group. On the other hand, it has been observed by analytical centrifugation of labelled melittin (10 μM) that large aggregates are pelleted at low speed, meaning that massive self-association must occur in such conditions.

Finally, the lytic activity of DNC-melittin and native melittin on human red blood cells were compared. Fig. 4 shows that the labelled peptide is more active by about a 3-fold factor.

4. DISCUSSION

That is the second time melittin is successfully modified with incorporation of a highly fluorescent dansyl group, since a very recent report [21] documents the behaviour in solution of a synthetic melittin bearing a dansyl probe at the N-terminus. In our study, it is linked through cadaverine moiety with TGase and results in a single DNC located on Gln_{25}. This derivative has a very high propensity to self-associate in buffer even at much lower concentration than native melittin [20]. This DNC-melittin proved to be more powerful for inducing erythrocyte lysis, as compared with native peptide.

This suggests that increasing significantly hydrophobicity away from the Lys_{21}-Arg-Lys-Arg_{24} cluster of charged residues improves both the stability of amphipathic aggregates in buffer and the activity. This fits with the early melittin derivative reported to have a higher activity and also to be self-associated [22]. These interesting properties make DNC-melittin a good tool to further investigate the peptide when bound to biological membranes.

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