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Acyl Transfer from Phosphocholine Lipids to Melittin

Catherine J. Pridmore,[a] Jackie A. Mosely,[a] Alison Rodger[b] and John M. Sanderson*[a]

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Transfer of fatty acyl groups from membrane phospholipids to melittin, a commonly studied membrane-active peptide, has been observed to occur over extended time periods. Transfer can be detected after 1-2 days and selectively targets amino groups at the N-terminal end of the peptide.

Chemical modifications to peptides and proteins, most notably the additon of acyl groups such as myristoyl or palmitoyl, are known to produce profound changes to the membrane behaviour of these molecules, with effects including increased membrane affinity and modified partitioning behaviour.[1-3] Acyl groups are usually added enzymatically *in vivo* (both translationally and post-translationally).[3] In the absence of enzyme catalysis, the membrane itself is generally seen as an inert medium. Despite the large number of membrane-active peptides that are studied for their intrinsic membrane behaviour, or as models for proteins, the chemical purity and identity of peptide-lipid systems is seldom examined upon the completion of binding experiments. Most biological membranes have a significant protein content (typically between 20% and 80% by weight)[4] and proteins may be in contact with the membrane for a considerable period of time before recycling, with half-lifes ranging from minutes to days.[5,6] As a consequence, studying the longer term behaviour of peptide- and protein-lipid systems is of fundamental interest, particularly with regard to examining the reactivity of these systems towards reactions such as acyl transfer from the lipids to the protein.

Melittin (H-GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) is a widely studied membrane active peptide, with well-documented pore-forming properties.[7] During the course of experiments to examine the effects of melittin on lipid stability,[8] we undertook control experiments using synthetic melittin in order to circumvent the possibility of complications due to any activity of the enzyme phospholipase A_2 (PLA_2), which is usually co-purified with melittin prepared from bee venom.[9] Addition of synthetic melittin to liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in phosphate buffered saline (PBS) at 37 °C and pH 7 led to the formation of new species, detectable in the mass spectrum of the sample (Fig. 1A) after a period of 1–2 days. These new species are identified as melittin plus the addition of an oleoyl chain, observed as protonated and sodiated adducts. When incubated under similar conditions with liposomes prepared using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), species were observed after 2 days corresponding to the transfer of either an oleoyl or a palmitoyl group from the lipid to the peptide (Fig. 1B), again as protonated and sodiated adducts.

Further analysis by TLC and mass spectrometry revealed the presence of *lyso*-phosphatidylcholine (*lyso*-PC) in the mixtures, indicating that the reaction to produce the acylated melittin species was likely to involve attack of a reactive nucleophile on the peptide with the carbonyl groups of the lipid esters. In order to monitor the progress of the reaction, the experiments were repeated and the formation of *lyso*-PC was followed by mass spectrometry as an indicator of the extent of reaction. Experiments were conducted at a molar peptide:lipid ratio of 1:5. Although this ratio is at the upper end of the range typically used for membrane binding experiments, at lower peptide concentrations it proved challenging to detect the formation of small quantities of the reaction products in the presence of a large excess of lipid.

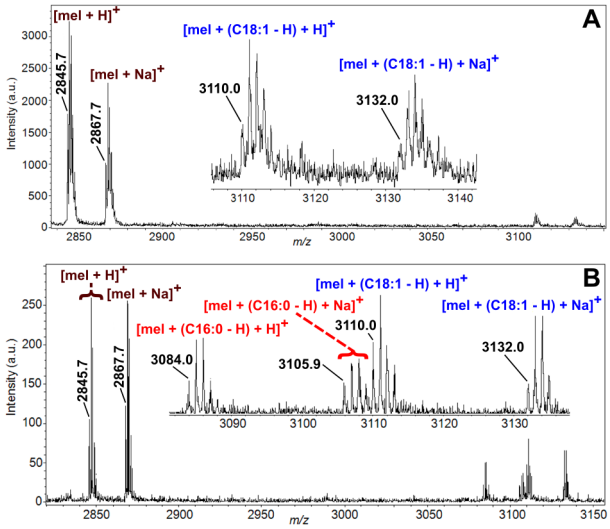

Fig. 1 Mass spectrum of melittin following mixing with DOPC (A) and POPC liposomes (B) at 37 °C in PBS (8 mM phosphate, 123 mM NaCl) at pH 7. Melittin and lipid concentrations were 71 μM and 0.35 mM respectively. Mass spectra were obtained after 14 days (A) or 28 days (B) using matrix-assisted laser desorption ionisation (MALDI) with α-cyano-4-hydroxycinnamic acid (CHA) as matrix. Ions at *m/z* 3110.0 and 3132.0 are assigned as [melittin + (oleoyl – H) + $H]^+$ and [melittin + (oleoyl – H) + $Na]^+$ respectively (calculated monoisotopic *m/z* 3110.0 and 3132.0); ions at *m/z* 3084.0 and 3105.9 are assigned as [melittin + (palmitoyl – H) + $H]^+$ and [melittin + (palmitoyl – H) + $Na]^+$ respectively (calculated monoisotopic *m/z* 3084.0 and 3106.0).

Even at a molar peptide:lipid ratio of 1:5, due to the excess of lipid it was necessary to remove and concentrate (by lyophlisation) a significant volume of the sample in order to detect the *lyso*-PC by TLC. With DOPC, in the initial stages (Fig. 2) the reaction was slow, with the first statistically significant detection of *lyso*-PC after 2 days. The intensity of the *lyso*-PC peaks increased steadily over the next 14 days and

then increased slowly thereafter. No formation of *lyso*-PC was observed in control experiments without melittin and oleic acid was not detected in any of the samples (with or without melittin), indicating that the level of background lipid hydrolysis was negligible. Due to absence of either competing hydrolysis or multiple acylation products, the formation of *lyso*-PC was therefore taken to be a reliable indicator of the extent of melittin acylation.

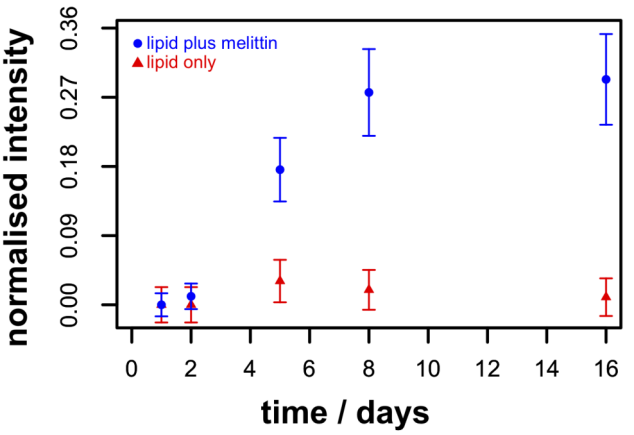

Fig. 2 The formation of *lyso*-PC monitored by MALDI-MS following mixing of melittin (71 μM) with DOPC liposomes (0.35 mM) in PBS at 37 °C and pH 7. MALDI-MS was performed using 2,5-dihydroxybenzoic acid as matrix. Normalised peak intensities for *lyso*-PC are calculated as the sum of the intensities of the protonated and sodiated *lyso*-PC peaks divided by the sum of the intensities of the protonated and sodiated *lyso*-PC and DOPC peaks. Errors are estimated from repeat experiments.

After 16 days, the relative intensity of the *lyso*-PC peaks was 0.3 ± 0.05. By comparison with a 1-oleoyl-*sn*-glycero-3-phosphocholine/DOPC calibration curve, this corresponded to a mole fraction of *lyso*-PC in the sample of 0.1 ± 0.02 and a corresponding extent of conversion of the melittin to acylated product of 50 ± 10%. This compared favourably with estimation of the extent of *lyso*-PC formation by TLC, which also indicated an extent of conversion of 50 ± 10% after 17 days (see the ESI). In experiments with POPC, similar kinetic profiles were obtained, with relative *lyso*-PC peak intensities of 0.15 for palmitoyl-*sn*-glycero-3-phosphocholine (corresponding to oleoyl transfer) and 0.07 for oleoyl-*sn*-glycero-3-phosphocholine (corresponding to palmitoyl transfer) after 14 days, and corresponding values of 0.18 and 0.10 after 28 days (see the ESI). These data indicated that acyl transfer from POPC was slower than from DOPC, and transfer of the oleoyl group was favoured over the palmitoyl group.

The most favourable positions of melittin for nucleophilic attack could reasonably be proposed as the N-terminal amino group and the ε-amino groups of the three lysines (K7, K21 and K23). Tandem mass spectrometry (MS/MS) was therefore performed on unmodified melittin and acylated melittin precursor ions in order to generate fragments that would permit the site of acylation to be localised. Performing all of these MS/MS analyses on the same sample, under identical conditions, enabled direct comparisons to be made between them. MS/MS of peptides generally induces cleavage along the backbone. Products of such cleavages are labelled as a-, b- or c-type should the fragment retain the N-terminus, and x-, y- or z-type should it retain the C-terminus.[10] In our case, a key requirement for confirming acylation at a particular site was the ability to identify unique product ions that only matched modification at that site and were therefore absent from the spectrum of the unmodified peptide. Furthermore, for reaction at a melittin amino group leading to amide formation, it was to be expected that cleavage of the acyl group would occur under MS/MS conditions, resulting in the formation of ions with similar or identical masses to the unmodified peptide. This was confirmed in the product ion spectra of both modified forms of melittin (*m/z* 3083 and 3110), in which an ion corresponding to loss of the acyl group was observed at *m/z* 2845. As a consequence, ladders of unmodified ions at the N-terminus or C-terminus of the peptide were unreliable as a means of identifying sites of modification.

In the product ion spectrum of oleoyl-melittin (*m/z* 3110), a ladder of b-type product ions can be identified that corresponds to N-terminal modification (Fig. 3a), although the first ion in this series (corresponding to the oleoylated N-terminal Gly) is absent. Nevertheless, the ions with *m/z* 435.3, 492.2 and 563.2 are unique to this spectrum and can be assigned as oleoylated fragments with confidence. These N-terminal fragment ions are consistent with the addition of an oleoyl group to the N-terminus of the peptide. A fragment with *m/z* 758.4 is also unique to this spectrum and matches a b-type N-terminal fragment that has lost ammonia (oleoyl-GIGAVL, b–17-type). An additional b-type fragment is found at *m/z* 1000.3 (oleoyl-GIGAVLKV) and a fragment assigned as an a–18-type at *m/z* 1069.5 (oleoyl-GIGAVLKVL). These latter two product ions are again consistent with N-terminal modification, but may also arise through modification of K7. Interestingly, the high-intensity ion with *m/z* 492.2 is also a match for an internal fragment with K7 oleoylation (oleoyl-KV, formed *via* b/y-type cleavage). A corresponding ion at *m/z* 464.1 matches both an a-type fragment (oleoyl-GIG) and an internal fragment (oleoyl-KV, formed *via* a/y-type cleavage). Weak ions at *m/z* 888.7 and 808.3 match internal fragments that include only K7 (oleoyl-AVLKVL and oleoyl-KVLTT), although the latter of these is also present in the spectrum of the unmodified melittin (Fig. 3c). None of the higher mass fragments enabled the position of modification to be identified with certainty. Taken together, it is clear that the N-terminal amino group is a significant site of modification, with likely additional modification of the side chain of K7. No evidence could be found for the addition of oleoyl groups to the remaining two lysines (K21 and K23).

Fragmentation of the palmitoylated melittin ion at *m/z* 3083 yields an N-terminal ladder of b-type ions at *m/z* 296.1, 409.2, 466.2, 537.2 and 636.3. Of these, the ions at *m/z* 409.2, 466.2 and 537.2 are unique to this spectrum. As with the product ion spectrum of oleoylated melittin, the peak at 466.2 can also be assigned as a palmitoylated internal fragment (palmitoyl-KV in this case). An additional fragment unique to this spectrum, at *m/z* 782.1 is assigned as an internal fragment involving modification of the side chain of K7 (palmitoyl-KVLTT formed *via* b/y-type cleavage). A number of product ions in this spectrum correspond to oleoylated products (*e.g.* the ion at *m/z* 492.2), which indicates that isolation of the parent ion

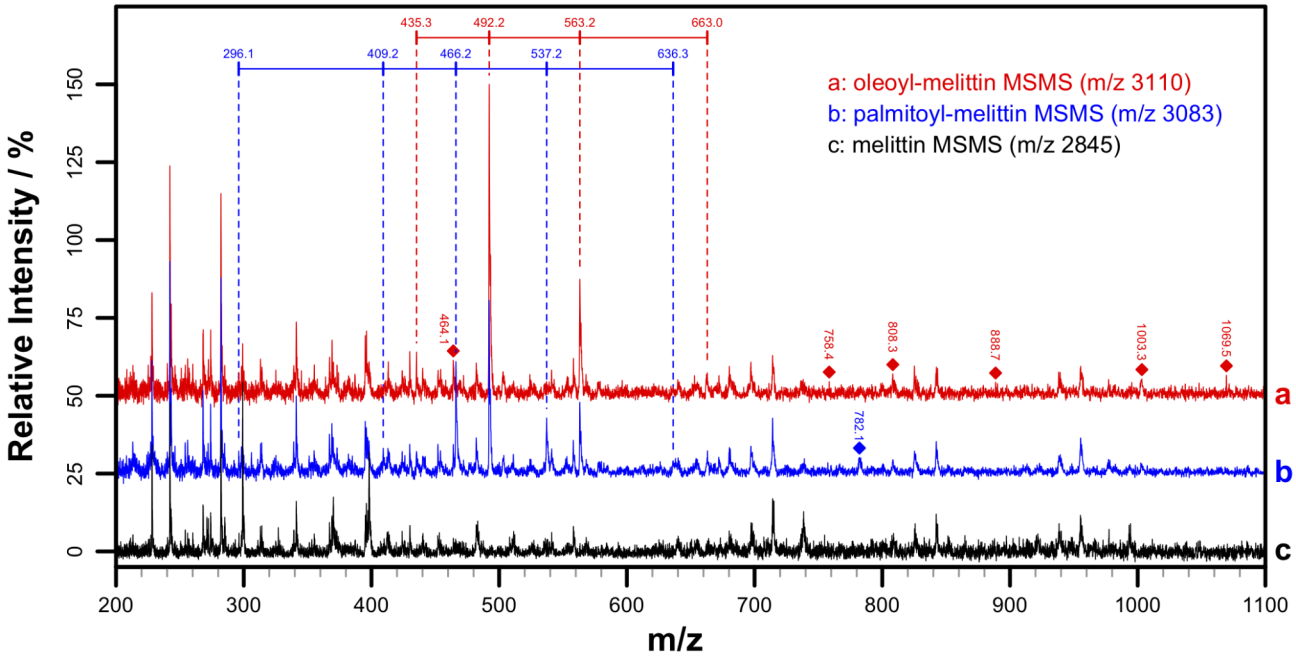

Fig. 3 A section of the MALDI-MS/MS spectra of (a) oleoyl-melittin (*m/z* 3110, offset on the *y*-axis by 50%), (b) palmitoyl-melittin (*m/z* 3083, offset on the *y*-axis by 25%) and (c) melittin (*m/z* 2845) from a sample of melittin incubated with POPC in PBS for 28 days at 37 °C, pH 7. Ladders corresponding to modified b-type fragments are indicated by dashed vertical lines. Key ions corresponding to modified (or potentially modified) fragments are indicated by diamonds. Spectra were obtained using CHA as matrix and are base peak normalised. See the ESI for full spectra and assignments.

was not complete. Nevertheless, these ions do not prohibit the identification of unique palmitoylated fragments. It may therefore be concluded that palmitoyl modification occurs at the N-terminal amino group, with the side chain of K7 likely to be an additional site of modification. No acyl melittin could be detected when peptide:lipid ratios were lowered to 1:50, although this may be more a consequence of a lack of relative sensitivity of the analytical methods than a lack of reaction. A number of other experiments were conducted in order to examine the effects of salts and metal ions on the acylation process. Repeating the experiments with DOPC in the presence of zinc or calcium salts gave data comparable to the PBS/NaCl data. Conducting the experiments with DOPC in phosphate buffer alone (*i.e.* without NaCl or any other metal salts) yielded no detectable oleoylated products. This latter result may reflect either a change in reactivity, or salt-related effects on melittin conformation.[11]

Overall, it is apparent that the amino groups of a prototypical membrane-active peptide are able to react with the ester groups of phosphocholines to yield product amides with an acyl group appended to the peptide. In the case of melittin at least, this process is selective for amino groups near the N-terminus of the peptide. The rate of acylation, although slow, is nevertheless relevant for membrane proteins that exhibit low rates of turnover in biological membranes. The ratio of peptide to lipid used, although higher than normal for many peptide binding experiments, is still within the range found for proteins in biological membranes, and as a consequence raises the possibility that similar processes may occur *in vivo*. It should be noted that it is unclear whether this reactivity of melittin is typical of all membrane peptides (and proteins), or the result of a particular chemical or structural feature of the peptide that favours reaction. It is perfectly feasible that this reaction will be promoted by the presence of particular amino acids near the sites of acylation and the scope of this reaction therefore merits further scrutiny.

Notes and references

[a] *University Science Laboratories, Department of Chemistry, South Road, Durham, DH1 3LE, UK. Tel: +44 (0)191 3342107; E-mail: j.m.sanderson@durham.ac.uk*

[b] *University of Warwick, Department of Chemistry, Coventry, CV4 7AL, UK. Tel: +44 (0)24 7652 3234; E-mail: A.Rodger@warwick.ac.uk*

† Electronic Supplementary Information (ESI) available: MS/MS data, TLC data, POPC kinetics, full methods. See DOI: 10.1039/b000000x/

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