Far From Inert: The Consequences for Cell Biology of the Intrinsic Reactivity of Lipids in Membranes

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Summary/Abstract
In this article it is hypothesised that a fundamental chemical reactivity exits between some non-lipid constituents of cellular membranes and ester-based lipids, the significance of which is not generally recognised. Many peptides and smaller organic molecules have now been shown to undergo lipidation reactions in model membranes in circumstances where direct reaction with the lipid is the only viable route for acyl transfer. Crucially, drugs like propranolol are lipidated *in vivo* with product profiles that are comparable to those produced *in vitro*. Some compounds have also been found to promote lipid hydrolysis. Drugs with high lytic activity *in vivo* tend to have higher toxicity *in vitro*. Deacylases and lipases are proposed as key enzymes that protect cells against the effects of intrinsic lipidation. The toxic effects of intrinsic lipidation are hypothesised to include a route by which nucleation can occur during the formation of amyloid fibrils.

1. Introduction

In their seminal paper on the Fluid Mosaic Model of the Structure of Cell Membranes, Singer and Nicolson stated that *"…the phospholipids and proteins of membranes do not interact strongly; in fact, they appear to be largely independent*".[1] This statement is increasingly being challenged by the discovery of numerous processes that involve direct participation by membrane lipids. In addition to the non-covalent interactions that result, for example, in the formation of a boundary layer of lipids around a membrane protein[2] or changes to membrane curvature,[3] an accumulating body of evidence indicates that the chemical stability of the lipids that constitute the membrane can be significantly affected by the presence of non-lipid components in the membrane. Lipids that have ester-linked fatty acyl chains, such as diacylglycerophospholipids, have a surprisingly high intrinsic susceptibility towards lytic reactions involving nucleophilic attack at the carbonyl group. Both the catalysis of lipid hydrolysis by non-lipid components, leading to the formation of fatty acids and lysolipids (Figure 1),[4–6] and direct reactivity leading to fatty acyl group transfer from the lipid to the non-lipid component (*i.e.* a lipidation reaction), also with the concomitant formation of a lysolipid, [4,7–10] have been described. The term 'intrinsic lipidation' is used to describe this direct acyl transfer from a membrane lipid as the donor to a membrane-associated acceptor molecule.

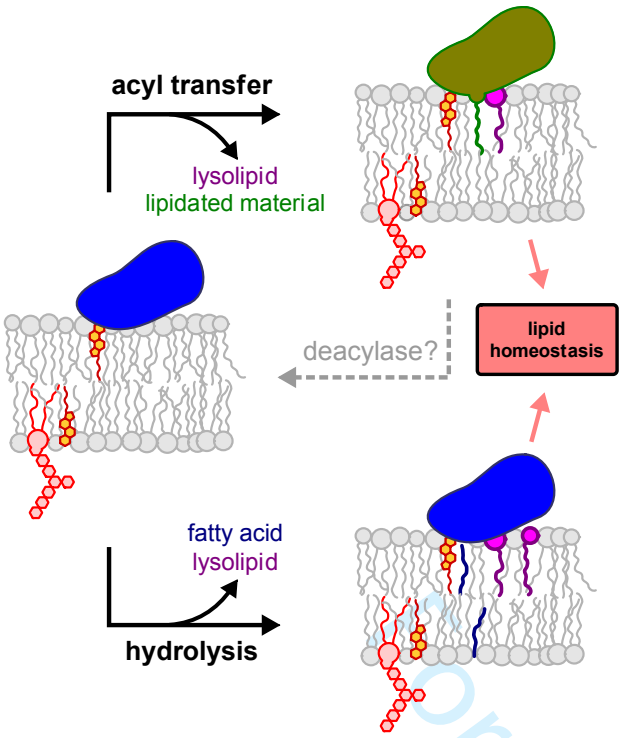

Figure 1. An overview of the reactions of ester-based lipids induced by non-lipidic components of the membrane. Acyl transfer from the lipid leads to intrinsic lipidation of the membrane-associated acyl acceptor in the leaflet to which the material is proximal, with the concomitant formation of one equivalent of lysolipid. Hydrolysis is catalytic, leading to the formation of multiple equivalents of fatty acid and lysolipid. Flip-flop of fatty acids between leaflets is usually much faster than that of lysolipids.[11] Adapted from https://commons.wikimedia.org/wiki/File:Cell_membrane_scheme.svg (CC-BY-SA-3.0-migrated).

Salient hallmarks of intrinsic lipidation are: the presence of a broad spectrum of acyl modifications to the acceptor that reflect the fatty acid profile of the ester-based lipids in the membrane leaflet(s) in which the process occurs; and incomplete conversion, with residual unmodified acceptor remaining present. There is generally little selectivity for transfer from the *sn*-1 *vs* the *sn*-2 position of glycerol. The consequences of this generation of extrinsic lipidated material are difficult to predict and will depend to a large extent on specific molecular properties. In many cases, the addition of an acyl group is likely to endow the material with surfactant properties and increase the residency of the material in the membrane. Both lysolipids and fatty acids are known to have profoundly disruptive biological effects when generated in excess, including cytotoxicity and membrane permeability enhancement.[11]

Lytic reactions of the kind outlined in Figure 1 potentially involve any molecule that is able to interact with a lipid membrane, regardless of size. The potential consequences of this reactivity therefore span a broad spectrum of biology, ranging from drug pharmacokinetics and mechanisms of toxicity for low molecular weight materials such as airborne pollutants, through disease mechanisms involving peptides, to post-translational changes to protein structure and function.

2. Lipidation Reactions Have Been Modelled *In Vitro* and Detected *In Vivo*

Lipidation reactions have been described in most detail for the peptide melittin using liposomes as a membrane model.[7,9,10] Other peptides are known to be reactive, including magainin II, penetratin and PGLa.[8] Typically, the sites to which acyl transfer occurs

include the N-terminal amino group, the ε-amino group of lysine, and the hydroxyl group of serine. Intrinsic lipidation exhibits selectivity not only in terms of the types of residue in the peptide structure that are modified, but also on their position within the structure. In the case of melittin for example (Figure 2), lipidation occurs predominantly at the N-terminus and the side chain of Lys23, although other sites are modified to a lesser extent, including Lys7, Lys21 and Ser18.[9] A common feature that links the predominant sites of modification is that they are predicted to reside at the interface between the hydrophilic and hydrophobic faces of the membrane-associated amphipathic helix.[8]

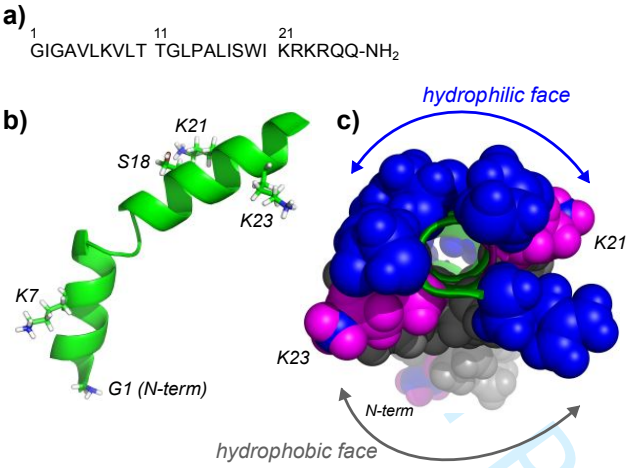

Figure 2. Melittin sequence and structure. a) Sequence. b) NMR structure (PDB entry: 2MLT). Residues that are modified by intrinsic lipidation are shown as sticks. c) View down the helix axis with the C-terminus closest to the viewer. Lysine residues are shown in magenta, hydrophilic residues in blue and hydrophobic residues in grey.

The intrinsic lipidation behaviour of membrane-active peptides is highly sensitive to membrane composition. For example, following the addition of melittin to membranes composed of the neutral lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), approximately 20% of the molecules are lipidated after 24 h. The inclusion of negatively charged lipids, phosphatidylethanolamine (PE), or cholesterol significantly increases reactivity. When 20 mol% cholesterol is included in POPC membranes, more than 50% of the melittin sample is lipidated within 24 h, despite melittin having a lower binding affinity for these membranes. The presence of cholesterol also changes the relative selectivity of N-terminal *vs* Lys23 lipidation in comparison to the same system without cholesterol.[7]

To date, studies of peptide lipidation have been conducted *in vitro* using liposomes as model systems. Liposomes offer the advantage of enabling conditions to be precisely controlled and systematically varied, without the complications that arise from the presence of other potential acyl donors, such as acyl CoA derivatives. Limitations of this approach, when compared to the situation *in vivo*, are that: products in the model system are not turned over and consequently accumulate and potentially influence subsequent reactivity; experiments are often conducted at higher drug:lipid ratios than used *in vitro*; and due to the lack of other components of cell membranes such as proteins, and the absence of dynamic changes to membrane composition that can occur as a result of homeostasis, idealised conditions *in vitro* might not always accurately model the situation *in vivo*. However, all of these limitations are mitigated by recent data obtained from a study of drug reactivity in both model and cell membranes where, at physiologically relevant concentrations, similar product profiles were found *in vitro* and *in cellulo* in Hep G2 cells.[4] In this work it was found the drug propranolol undergoes lipidation predominantly on the central hydroxyl group in a process that is formally a transesterification reaction with the lipid (Figure 3). The predominant products detectable in Hep G2 cells after 24 h are

propranolol modified with the following acyl chains: C16:0 > C18:1 > C18:0 > C16:1 ~ C14:0, with C18:2, C20:1, C20:2, C20:3, C20:4 and C22:6 fatty acyl modifications also detectable as minor products.

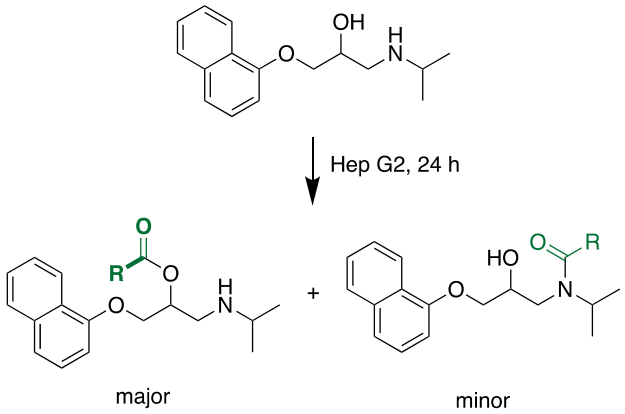

Figure 3. Lipidated propranolol species formed 24 h after administration of propranolol to Hep G2 cells. RC=O includes myristoyl, palmitoyl, palmitoleoyl, stearoyl and oleoyl, plus other minor products.

Hydrolytic reactions are particularly prominent for cationic amphiphilic drugs, where the protonated form of the drug in the membrane interface is proposed to act as an acid catalyst for lipid hydrolysis.[5] Whilst detection of lipid hydrolysis is well established in liposome models,[6] there is currently no direct evidence for lysolipid formation *in vivo*. However, compounds that lead to significant lysis in liposome models also tend to have a lower EC_{50} for the lysosomal storage disorder phospholipidosis,[4] so there is some indirect evidence for a link between hydrolytic activity and biological activity. It will be of interest to probe whether other lysosomal storage diseases have a link to membrane chemical reactivity.

3. Hypothesis: All Ester-based Lipids in Cellular Membranes Have a High Susceptibility to Lysis.

On consideration of the features of the intrinsic lipidation and hydrolysis reactions described above, the following hypothesis is proposed: *all ester-based lipids in cellular membranes have a high susceptibility to lysis and this reactivity is enhanced by the presence of organic molecules with particular structural features. Lysis may be catalysed by the organic molecule, or be the result of direct chemical reaction between the organic molecule and the lipid*.

On the basis of the data from the systems studied to date, some predictions may be made concerning the outcomes of this lytic reactivity should it occur *in vivo*:

1. The reactions will be very sensitive to changes in lipid and cholesterol composition and may therefore change significantly in situations where normal lipid homeostasis is disrupted.
2. Lipidation will produce products with both saturated and unsaturated fatty acyl groups due to a low *sn*-1/*sn*-2 selectivity.
3. For each lipidation site, a series of products should be present with a relative abundance that mirrors the fatty acid composition of the membrane leaflet in which the lipidated products form.
4. The lipidation process will be incomplete: unmodified material will be present.

5. The presence of lysolipids formed by either hydrolysis or lipidation will perturb the lipid profile of the host cell.

If, as hypothesised, these lipid-lytic reactions are more prevalent than generally recognised, immediate questions arise concerning the reasons why their products are not more readily detectable and why they are not significantly deleterious to cell function. The answers to these questions may lie in the number of challenges to detecting these lytic processes *in vivo*:

1. Even when experiments are conducted in ideal conditions *in vitro* the amounts of lysolipid formed are often small relative to bulk lipid, particularly in typical scenarios where reactions do not go to completion, requiring the use of sensitive analytical methods. The detection of lysolipid products *in vivo* is likely to be even more challenging as a consequence of the likely perturbations to lipid homeostasis that follow from the formation of lysolipids. Chemical reactivity may actually appear as changes in the abundance of other lipid classes. For example, in the propranolol study there is some evidence that the relative abundance of plasmalogens was increased in Hep G2 cells after administration of propranolol.[4]
2. Detection of lipidated products is challenging due to the diversity of products formed, combined with the likelihood of an incomplete reaction. In many cases, lipidated products evade detection because they are insoluble or elute in the wash phase at the end of a proteomics run. For example, N-terminal lipidated fragments of aquaporin-0 were found in mobile phases with >95% acetonitrile during LC-MS analyses of tryptic digests of the protein.[12]
3. It is difficult to predict the likely sites of lipidation on any molecule because the reactivity is structure-based, depending to a significant degree on the relative positioning of the molecule with respect to the membrane. Currently, for peptides and proteins it is hugely problematic to use sequence-based methods to predict intrinsic lipidation sites as they do not correspond to enzyme consensus sequences.
4. Post-translational modifications by intrinsic lipidation will in some cases lead to other complications during protein digests. For example, internal lysine residues modified by acyl groups lead to missed cleavages in tryptic digests alongside mass increases (from the additional acyl groups) for the intact fragment.[9,12]
5. Lipidiation at other internal sites, such as the thiol group of cysteine, the hydroxyl group of serine and threonine, the imidazole of histidine and the guanidine of arginine will be difficult to detect due to the reasons presented above added to a higher tendency for these modifications to be hydrolysed during sample preparation and more facile in-source fragmentation during analysis by mass spectrometry.[9]
6. When considered *in vivo*, it may be difficult to rule out lipidation from other acyl donors, including acyl coenzyme A (CoA) derivatives. For both the propranolol and aquaporin work described above, it is difficult to eliminate completely the possibility that lipidation occurred from acyl CoA.[13–15]

Cells are likely to have evolved mechanisms to counter this reactivity. In the case of both hydrolysis and intrinsic lipidation, the lysolipid product will be subject to normal lipid homeostatic mechanisms,[16] as will the fatty acids formed by hydrolysis, being recycled for example into *de novo* synthesised lipids. Reversal of internal lipidation is discussed below.

4. Reversal of Acylation by Deacylases and Lipases.

Proteins modified at an internal lysine by intrinsic lipidation would require a deacylase activity to reverse the modification (Figure 1). Examples of both Class I and Class III

deacylases are known with the ability to catalyse the removal of long chain fatty acyl groups attached to lysine. Of the Class I enzymes, the histone deacetlyase HDAC8 was shown to remove fatty acyl modifications up to 14C in length (myristoyl) from an internal lysine of a model peptide.[17] In this case, the catalytic efficiency was greater for the removal of longer chain acyl groups than for acetyl.

Of the Class III enzymes, several of the sirtuins are notable for possessing the ability to catalyse the removal of long acyl chains from internal lysine residues. Mammalian sirtuins are NAD^+-dependent enzymes and are classified in 7 homologous groups (SIRT1–7) that differ in their substrate specificity and subcellular localisation.[18–21] Although their activity is often discussed in terms of deacetylation, most have the ability to catalyse the lysis of longer chain fatty acyl groups from the side chain of internal lysine residues,[22,23] with Sir2A (*Plasmodium falciparum*)[24], SIRT2 (cytoplasm)[25] and SIRT6 (nucleus)[20,23] shown to have particularly high selectivity for demyristoylation. SIRT2 has been linked with regulation of the activity of some Ras proteins *via* control of the deacylation of an internal lysine side chain.[26,27] It is notable that some sirtuins, such as SIRT6, are associated with disorders in lipid and fat metabolism such as steatosis.[28]

With the increasing availability of simultaneous inhibitors for multiple classes of sirtuin (pan-inhibitors),[18,29,30] it should be possible to probe the hypothesis that they are involved in reversing lysine modification by intrinsic lipidation by examining the downstream effects of sirtuin inhibition on the accumulation of proteins modified at internal lysines. This would complement experiments where reduced sirtuin activity has been shown to lead to hyperacetylation of histone proteins.[31]

Several good candidates for enzymes generally capable of reversing lipidation are found amongst the metabolic serine hydrolases[32] These include acylprotein thioesterases (also known as lysophospholipases), which are able to catalyse the hydrolysis of internally acylated serines and are also associated with the hydrolysis of a broad spectrum of lysolipids.[32] One of these, acylprotein thioesterase 1 (APT1) is present mostly in the cytosol, but has also been detected in many cellular membranes. Reversal of palmitoylation has been suggested to be a key function of this enzyme,[33] but deacylation of serine esters has also been reported.[34]

The hypothesis presented above predicts that the lysis products may not be evident under normal circumstances, either because they are only ever present at low levels or because they are relatively non-deleterious. It stands to reason then that if circumstances arise where either lytic products form that are highly toxic, or the balance between the generation and removal of lytic products is disrupted, then the products will become apparent. Potential scenarios for each of these cases will be presented below.

5. Membrane Protein Lipidation in the Absence of Deacylase Enzymes

Evidence of the potential effects of disrupting the balance between the generation and removal of lytic products can be found in scenarios where deacylase enzymes are not present. For example, lens fibre cells are post-mitotic, from which much of the normal cellular organelles and associated enzymes are removed during terminal differentiation.[35] Aquaporin-0 (AQP0) is a major component of the lens fibre membrane and is lipidated at two sites in the protein, the N-terminus and Lys238 (Figure S1), neither of which is predictable on the basis of known consensus sequences for lipidation enzymes. Both sites are modified with an unusual combination of acyl groups including C16:1, C18:0, C20:1, C20:3, and C20:2 for bovine AQP0 and C16:1, C18:0, C20:1 and C20:4 for human AQP0.

Neither site is completely lipidated. This combination of features bears all the hallmarks of an intrinsic lipidation process.[12]

Another example may be found in pulmonary surfactant. This lipoprotein complex is rich in the lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and surfactant proteins. One of these proteins, surfactant protein C, is palmitoylated at up to three internal sites, two of which are cysteine with the remaining one being a lysine (Lys11).[36–39] This lysine is predicted to reside in the vicinity of the membrane interface in the transmembrane protein.[40,41] Lipidation of the lysine is incomplete, as expected for a modification arising by intrinsic lipidation, giving rise to a series of products with differing numbers of additional palmitoyl groups.[36]

6. Peptide Conformational Changes Driven by Lipidation May Lead to Toxicity

It is well established that lipidation of membrane proteins can drive local conformational changes and influence both the membrane affinity of a protein and its lateral distribution within the membrane.[42–44] Lipidation can have even more pronounced effects on peptides that, when compared to proteins, are more likely to be unfolded when non-lipidated due to their smaller size and undergo a more pronounced overall change in hydrophobicity (or amphiphilicity) following the addition of a fatty acyl group. A number of peptides are known for which the lipidated form has both an increased tendency to self-aggregate and a higher affinity for lipid membranes, leading in both cases to the adoption of secondary structure at concentrations where the non-lipidated form is a random coil. [45–52] Cases have also been documented where addition of an acyl group changes peptide aggregation behaviour and leads to the formation of fibrils rich in β-sheet. For example, short lysine-rich peptides derived from collagen adopt β-sheet conformations when palmitoylated and have a tendency to aggregate to form micelles or, in some cases, amyloid-like fibrils.[53,54] Amyloid-like fibril formation has also been reported for short lipidated peptides rich in β-branched amino acids.[55]

Several peptides that are able to form insoluble amyloid fibrils have a significant affinity for lipid membranes and in many cases this binding is known to facilitate fibrillogenesis, through factors such as changes in secondary structure to favour β-sheet conformations or on-pathway helical intermediates and increased local concentrations on the membrane. [56–64] In the absence of membranes, numerous partially folded conformations (or microstates) exist in equilibrium with misfolded conformations. In appropriate circumstances, some of these misfolded conformations can nucleate to form oligomers (Figure 4a, *path a*). [58,59,65–69] Subsequent elongation of these oligomers leads to fibrils, with the overall kinetics controlled by the associated rate for nucleation and elongation. Nucleation is usually the rate determining step and is responsible for the observation of a lag phase during fibrillogenesis. In the prevailing view, peptide binding to membranes drives conformational change towards on-pathway fibril-forming aggregation intermediates and increases local concentrations of peptide (Figure 4a, *path b*), thereby increasing the rate of nucleation. An alternative view presented here, consistent with the intrinsic lipidation hypothesis, is that peptide lipidation is able to achieve similar outcomes of increasing local conformation and driving conformational change towards on-pathway intermediates (Figure 4a, *path c*) and occurs during the lag phase. There are several reasons why this hypothesis is appealing:

1. It can account for several observations that lipids are incorporated into fibrils. [59,70–76] In cases where fluorescent lipids are used, the associated fluorescence of the fibrils could arise either from the formation mixed micelles of lipdated peptide and

lipid, or from direct transfer of a fluorescent acyl chain to the peptide during nucleation.

2. Nucleation by *path c* would be sensitive to a range of membrane-related factors that affect lipidation, such as surface potential, hydration (which is in turn affected by oxidation), and changes in lipidome as a result of ageing or disease,[77,78]. This sensitivity could account for numerous apparent contradictions or unexplained patterns of fibrillogenesis, such as the discrepancy between Aβ(1-40)[79] and Aβ(1-42) [80] nucleation rates in neutral membranes, variations induced by the method of sample preparation,[73,81] and extracellular amyloid deposition remote from the site of peptide or protein formation.[65,82]
3. The susceptibility to some amyloid diseases is inherited.[65,82] Intrinsic lipidation is sensitive to peptide interfacial orientation in the membrane. Changes in interfacial orientation or disposition might, for example, arise as a consequence of sequence changes that modify hydrophobicity or amphiphilicity. Lipidation is therefore able to provide a link between gene mutations and the susceptibility to amyloid formation.
4. The affinity of some amyloid-forming peptides for membranes with compositions representative of the plasma membrane is low.[80] Lipidation provides a mechanism to increase peptide affinity with these membranes.
5. Lipidated peptides associated with the membrane will be pre-oriented, have a high local concentration, and be able to diffuse freely in the plane of the membrane,[83] effectively transforming a 3D nucleation process into a 2D one with a much lower entropic energy barrier (Figure 4b).[84,85]

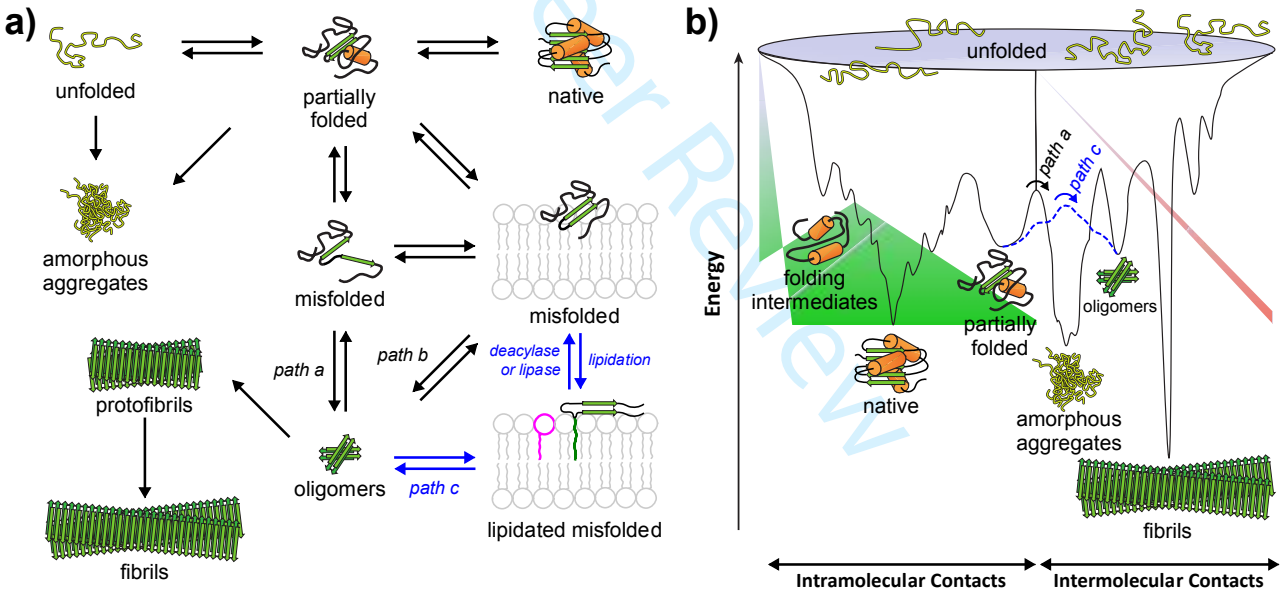

Figure 4. Protein folding pathways leading to the formation of fibrils. *Path a* is the nucleation pathway in the absence of membranes; *path b* is an alternative pathway in or on the membrane; *path c* involves facilitated nucleation by a low energy lipidated peptide formed by intrinsic lipidation. a) Schematic representation of key intermediates. Most aggregation intermediates comprise an ensemble of microstates. b) Energy level diagram. Some intermediates have been omitted for clarity. Adapted by permission from Springer Nature: *Nature*, Molecular chaperones in protein folding and proteostasis, Hartl *et al*., 2011.[86]

Three cases where it may be hypothesised that lipidation plays are role are outlined below. One of these, amyloid Aβ, is described in more detail in Box 1; the other two, α-

synuclein and Human Islet Amyloid Polypeptide, are discussed in more detail in the supporting information.

The aggregation of the Aβ peptides such as Aβ(1-40) and Aβ(1-42) to form amyloid fibres is a key process during the progression of Alzheimer's disease.[65,66,87] Aβ peptides are formed in neuronal cells by proteolytic processing of the amyloid precursor protein (APP) by γ-secretases (Figure 5).[84,88] APP has a single transmembrane helix and an extracellular hydrophilic domain. Many of the key sites cleaved by γ-secretases lie in the transmembrane region.[88,89] Recent work examining the conformation of Aβ(1-40) in lipid membranes has established that at low peptide to lipid ratios, Lys16, Ser26 and Lys28 adopt dispositions close to the membrane interface in a partially helical structure, with subtle variations in peptide structure exhibited in neutral membranes according to the level of chain unsaturation.[90]

The model shown in Figure 5a is proposed to account for a lipidation-based pathway for nucleation, whereby the cleavage of APP releases an Aβ peptide which retains some helical structure in solution[84,91–93] and undergoes reversible binding to the membrane that drives further structure formation towards an orientation that favours acyl transfer to the peptide at Lys16, Ser26 and Lys28. It is notable that Ser26 is in known to reside in the turn part of the β-structure[94] which would make this residue a good candidate for lipidation and presentation of the lipidated peptide in an orientation available to promote oligomerisation. Recent studies have also established that Ser26 and Lys23 are close to the membrane interface in the transmembrane topology of APP,[95] leading to an alternative pathway shown at the bottom of Figure 5a in which APP is lipidated before secretase cleavage. Appealing features of this shorter pathway are its brevity and the retention of the Aβ peptide in the membrane. The timescales for a lipidation-driven nucleation are reasonable, as even in systems with accelerated nucleation,[96] the nucleation half times are greater than one hour, putting them on the timescale that lipidation of melittin begins to be observable.[8,9]

Other cases where it may be hypothesised that intrinsic lipidation plays a role in nucleation include α-synuclein (α-syn) and Human Islet Amyloid Polypeptide (hIAPP). Deposition of α-syn in the central nervous system is associated with a number of disorders associated with ageing, including Parkinson's disease.[97,98] The lysines of a KTKEGV repeat sequence of α-syn, which lie at the boundary between the hydrophilic and hydrophobic surfaces of a helix (Figure 6b), are potential lipidation sites. Good candidate residues include Lys43, Lys45, Lys58 and Lys60, in addition to one serine (Ser42). These residues lie close to the membrane interfacial region.[97] Additionally, on the basis of a high sensitivity of fibril formation to lipid composition, the presence of additional minor conformations when fibrils are formed in the presence of membranes, and additional α-syn mass accrued by post-translational modifications,[99] it is proposed that intrinsic lipidation (Figure 4, *path c*) is a valid nucleation pathway for this protein. Fibril formation by hIAPP in the pancreas is a feature of type 2 diabetes.[100] Lipid binding accelerates fibril formation and it has been established that hIAPP exhibits cooperative binding to membranes in a process that involves nucleation.[101] NMR relaxation experiments place two serine residues (Ser19 and Ser20) of hIAPP as potential lipidation sites in the membrane interface close to the boundary between the solvent exposed and fatty acyl chain exposed sections of the peptide (Figure 6c).[102]

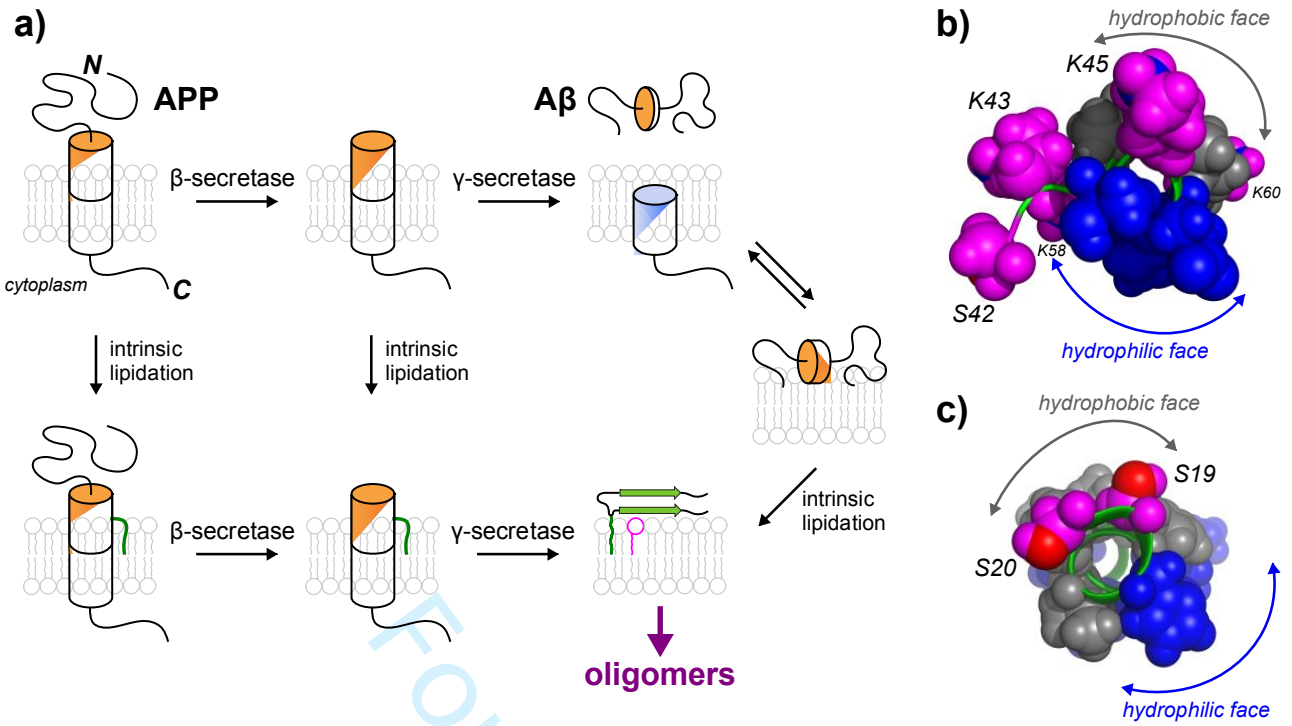

Figure 5. Proposed role of intrinsic lipidation in amyloid formation. a) Pathways for nucleation of Aβ peptides. Shown at the top, secretase cleavage of APP releases an Aβ peptide which undergoes reversible binding to the membrane in an orientation that favours acyl transfer to the peptide. An alternative route involving lipidation of APP before secretase cleavage is shown at the bottom. b) Residues 7 and 25 of the N-terminal section of α-syn (PDB entry 1XQ8 [103] viewed along the helix axis with residue 7 nearest the viewer. c) Residues 42–59 of hIAPP (PDB entry 2KB8), looking down the helix axis with residue 42 closest to the viewer. In b) and c), amphiphilic sequences from amyloid forming peptides. Lysine and serine residues are shown in magenta, hydrophilic residues in blue and hydrophobic residues in grey.

The three cases outlined above are examples where intrinsic lipidation is hypothesised to operate as a nucleation pathway for fibril formation, in addition to the solution and membrane-associated pathways (Figure 4, *paths a* and *b*). The lipidation pathway is not limited to these three cases however, and may account for the features of a number of other protein misfolding diseases. In cases where regulatory agents such as chaperones prevent nucleation by *paths a* or *b*,[104] lipidation may provide the only route to nucleation. Proteins relevant for this lipidation hypothesis have the common feature, along with melittin, of existing as wholly or partially unstructured peptides in solution that adopt an amphipathic helical structure in the membrane that places reactive residues of lysine or serine close to reactive lipid carbonyl groups in the membrane interface. Lipidation may drive the peptides towards on-pathway intermediates and increase their membrane affinity, thereby promoting increased rates of nucleation. It should be recognised that proving this hypothesis is a very significant challenge, as it is implicit that intrinisic lipidation will affect only a very small proportion of peptides in the fibrillar structure that are buried at the sites of nucleation, and the nucleating centres themselves will have similar backbone conformations to the rest of the fibril around them. The modifications themselves, being esters in the case of serine and secondary amides in the case of lysine, are likely to be difficult to resolve spectroscopically from lipid esters and backbone amides respectively.

As a final point, it is worth considering whether the deamidases and lipases discussed above in section 4 can provide the same protection against nucleation *via* lipidation (Figure 4, *path c*) as chaperones can against protein misfolding in solution (Figure 4, *path*

a). Interestingly, many sirtuins have been reported to offer protection against Alzheimer's disease and other neurodegenerative diseases that involve protein misfolding, such as Huntingdon's disease and Parkinson's disease.[105] Loss of SIRT6 activity is found in patients with Alzheimer's disease,[106] whereas by contrast increasing SIRT6 activity is able to protect against Aβ42-induced DNA damage.[107] SIRT1 is downregulated in Parkinson's disease, whereas SIRT1 over-expression reduces the formation of α-synuclein aggregates in a neuroblastoma cell line in conditions of oxidative stress known to lead to the formation of protein aggregates.[108]

Box 1: The Role of Membranes in Aβ Fibrillogenesis

Binding of Aβ peptides to membranes leads to various effects, including detergent-like activity with the formation of micelle-like structures,[70] ion channel formation, and interfacial activities such as membrane fusion and disruption according to a carpet model.[109,110] At low coverage (*i.e.* a low peptide:lipid ratio) there is evidence for helical and partially folded conformations.[68,90] The composition of the membrane exerts a significant effect on the interactions of Aβ with membranes,[78,111] with even changes in the neutral lipid content affecting binding.[89] Binding affinity increases with an increase in negatively charged lipids and is affected by the presence of the ganglioside GM1.[110]

The inclusion of cholesterol in neutral and negatively charged membranes *in vitro* changes the binding characteristics of most Aβ peptides. For example, at 20 mol%, cholesterol hinders interaction of Aβ(25-35) with neutral membranes composed of unsaturated lipids.[112,113] By contrast, for membranes composed of saturated lipids, high cholesterol levels facilitate deeper penetration of Aβ(1-40),[114] and significantly increase nucleation rates of Aβ(1-42).[96] The behavior of Aβ peptides *in vivo* in the presence of cholesterol is complex to disentangle, not least because cholesterol levels change with cellular location, age, disease, and under the influence of some medications.[115] Cholesterol levels also affect the activity of γ-secretases[87,116,117] and the distribution of APP.[118] In diseased brains, the cholesterol content of the temporal gyrus is significantly reduced when compared with non-diseased brains.[110] This reduction will effect Aβ binding to the membrane, changing not only the strength of the interaction but also the penetration depth. Our work with melittin indicates that penetration depth is the more significant of the two factors in determining the rate at which lipidation occurs.[7] As a whole, these data indicate the there is a very real potential for small changes in cholesterol levels or lipid composition to place suitable Aβ residues in a position to undergo intrinsic lipidation. Consistent with this notion, NMR studies using ^{13}C labelled amino acids incorporated into synthetic peptides suggest that Asp23 and Ser26 are close to lipid methylene groups.[73] The α-C atoms of Gly25 and Val36 have also been demonstrated to have close contacts with ^{31}P. [70]

There is some support for lipidated Aβ peptides being able to promote fibril formation. Short synthetically engineered fragments of amyloid Aβ palmitoylated on multiple lysine side chains adopt pathogenic fibrillar conformations in model membranes and are able to illicit an Aβ-specific immune response.[119,120] The β-sheet content of these peptides is influenced by the degree of lipidation, but in most cases they are able to assemble into fibrils in the absence of membranes.[121] Control peptides bearing acetyl groups in place of palmitoyl generally did not display this behaviour, remaining as random coil structures in solution. In some cases, the morphology of Aβ(1-40) fibrils formed in the presence of membranes has been found to differ from those formed in solution,[122] indicating that at least in some cases growing fibrils are able to access different intermediates in the fibrillation pathway.

7. Testing the Hypothesis

The discussion above enables some predictions to be made that can be used to test the hypothesis that lipids are highly susceptible to lytic reactions.

Prediction 1: proteins are lipidated by chemical reactions with membrane lipids. The main obstacle to overcome in order to test this prediction is to demonstrate that fatty acyl modifications could not have occurred *via* the corresponding CoA derivatives. A tangible approach would be to supply a labelled fatty acid to an organism and 'chase' the label as it distributes through the lipids to the protein. The inclusion of CoA synthetase inhibitors at differing times and duration would enable the source of lipidation to be narrowed.

Prediction 2: deacylases and lipases operate to correct proteins modified by intrinsic lipidation. As discussed above, proving this could be achieved, for example, by an observation of increased levels of lipidated proteins following the administration of pan-inhibitors of sirtuins.

Prediction 3: intrinsic lipidation plays a key role in some protein folding diseases. Direct proof of this involves identification of a small proportion of lipidated peptides within an insoluble fibre. There is a major obstacle in detecting minor modifications to an amino acid side chain that lie in the middle of an insoluble fibre. However, modern MS approaches such as ion mobility MS that offer improved methods for isolating minor species may offer an opportunity to detect these modifications.[123]

Prediction 4: lipid lysis generated by membrane-active agents is a factor in some lysosomal storage disorders. This prediction is challenging to demonstrate because the lysis of lipids generates lysolipids and fatty acids which, *in vivo*, will be redistributed by cellular homeostatic mechanisms and may or may not generate a subsequent adaptive response. One approach could be to probe lipid dynamics using pulse/chase experiments with a labelled lipid to prove that turnover increases in the presence of a lytic agent, combined with cell imaging to determine the temporo-spatial distribution of the pulse and identify its presence in structures such as lamellar bodies and lysosomes.

8. Conclusions

Intrinsic lipidation, involving direct acyl transfer from a lipid to an acceptor molecule, has been demonstrated *in vitro* in conditions where there are no possible competing processes that can involve enzymes or coenzyme A derivatives. It is therefore likely that conditions will exist under which this process will occur *in vivo*. The process should be particularly apparent where circumstances permit the accumulation of the intrinsically lipidated products, such as when protective measures to remove lipidated products are absent or the lipidated products themselves are toxic and/or insoluble. A significant conceptual challenge arises from the requirement to prove that acyl group modifications have arisen from the lipid rather than a coenzyme A derivative. It is nonetheless remarkable that molecules modified by multiple fatty acyl group modifications to a single site, ranging in size from whole proteins at one extreme to drug molecules at the other, have been isolated from biological samples. There are sufficient reasons to believe that both intrinsic lipidation and lipid hydrolysis catalysed by membrane-associated compounds could produce a number of detectable outcomes in cell biology.

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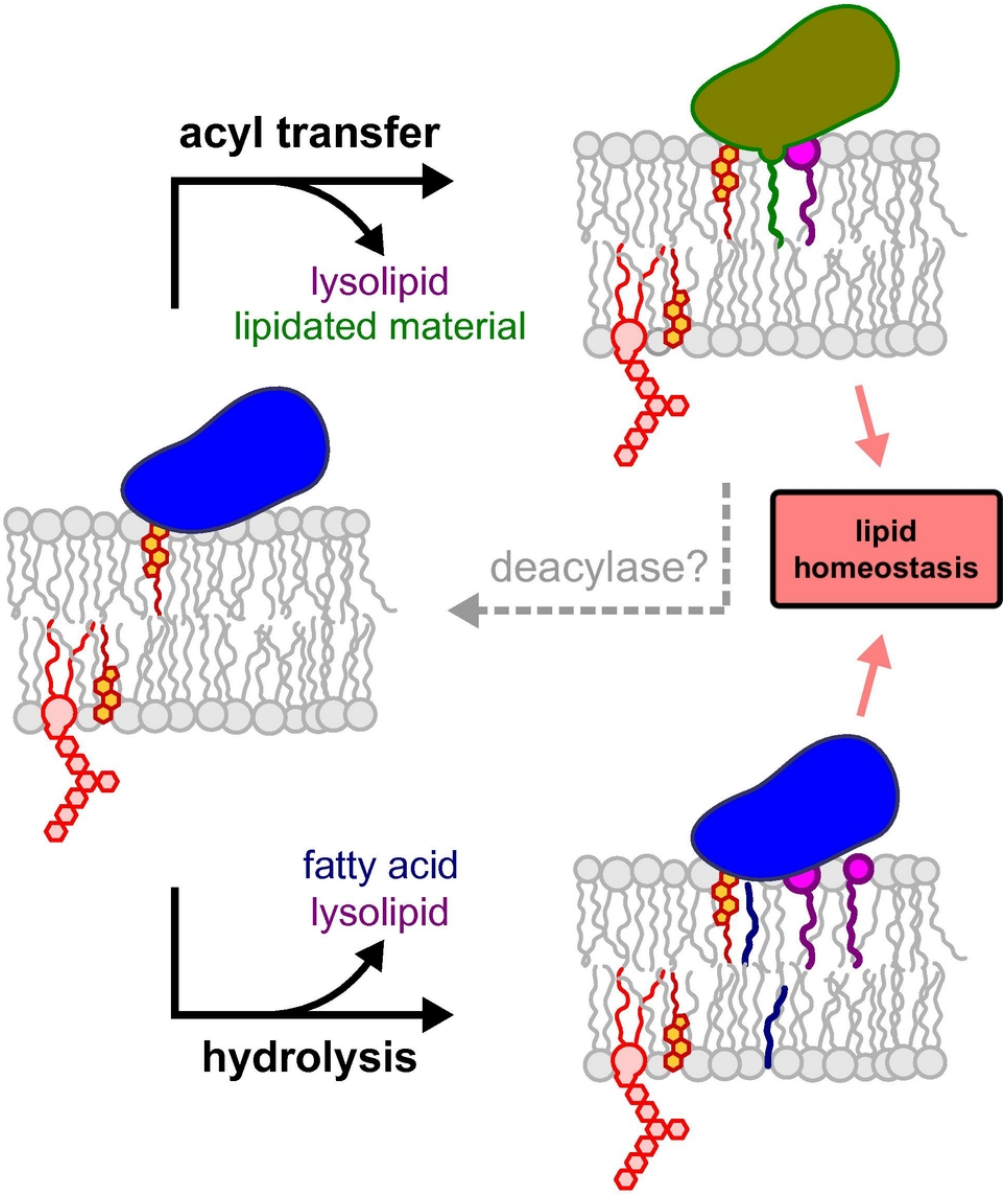
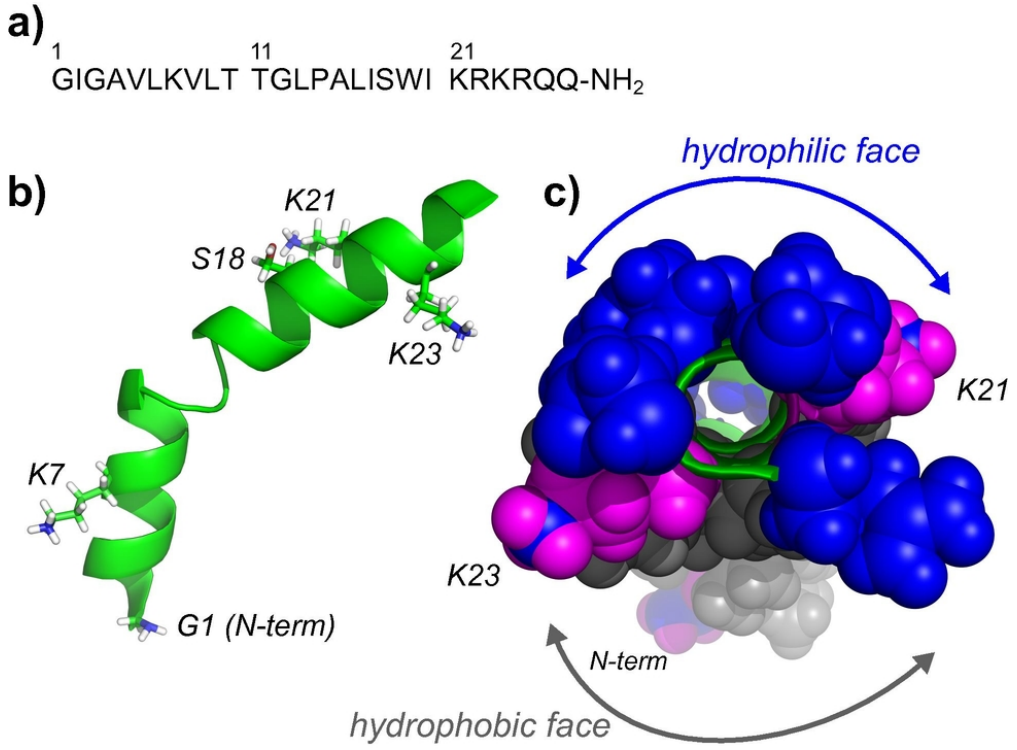


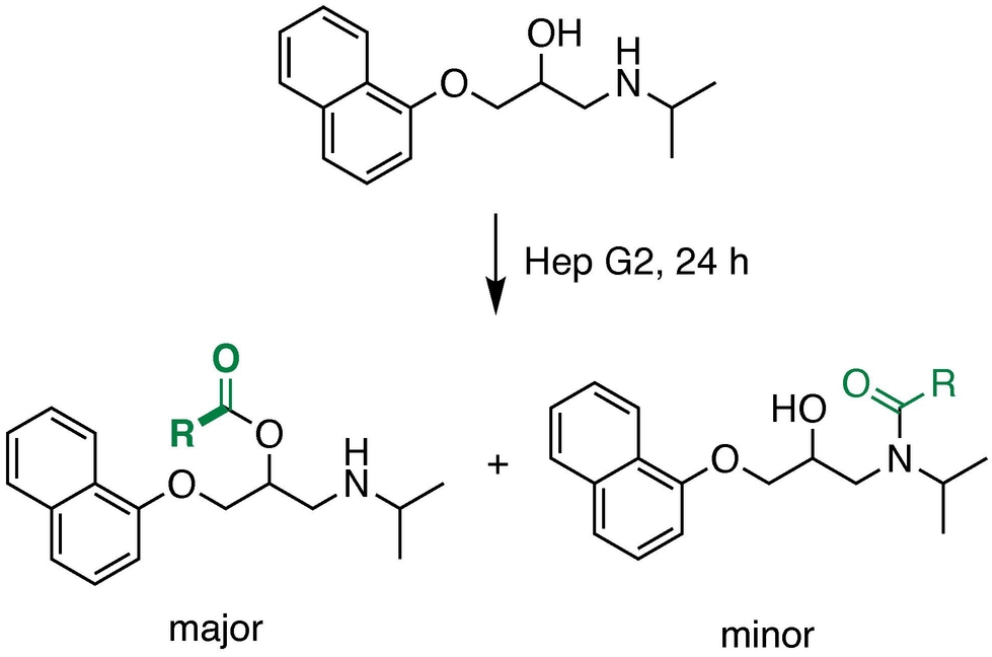
An overview of the reactions of ester-based lipids induced by non-lipidic components of the membrane. Acyl transfer from the lipid leads to intrinsic lipidation of the membrane-associated acyl acceptor in the leaflet to which the material is proximal, with the concomitant formation of one equivalent of lysolipid. Hydrolysis is catalytic, leading to the formation of multiple equivalents of fatty acid and lysolipid. Flip-flop of fatty acids between leaflets is usually much faster than that of lysolipids.[11] Adapted from https://commons.wikimedia.org/wiki/File:Cell_membrane_scheme.svg (CC-BY-SA-3.0-migrated).

81x96mm (300 x 300 DPI)



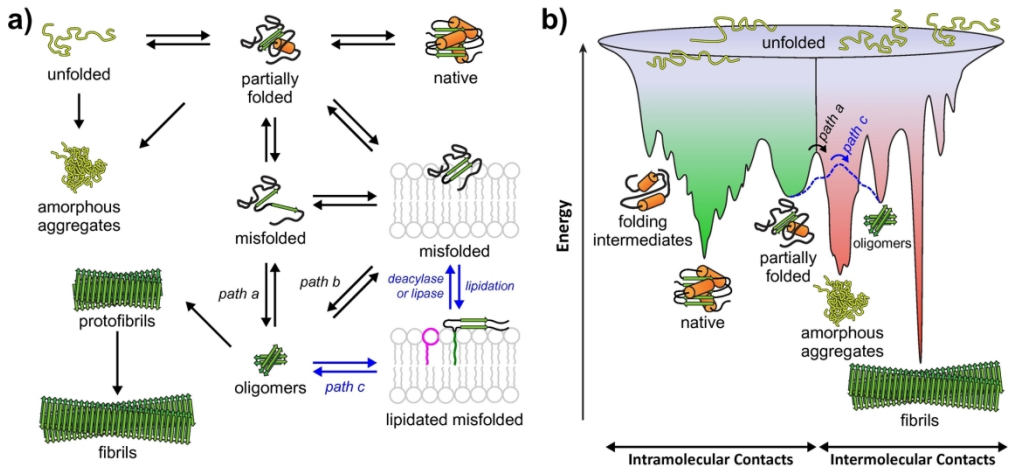
Melittin sequence and structure. a) Sequence. b) NMR structure (PDB entry: 2MLT). Residues that are modified by intrinsic lipidation are shown as sticks. c) View down the helix axis with the C-terminus closest to the viewer. Lysine residues are shown in magenta, hydrophilic residues in blue and hydrophobic residues in grey.

82x61mm (300 x 300 DPI)



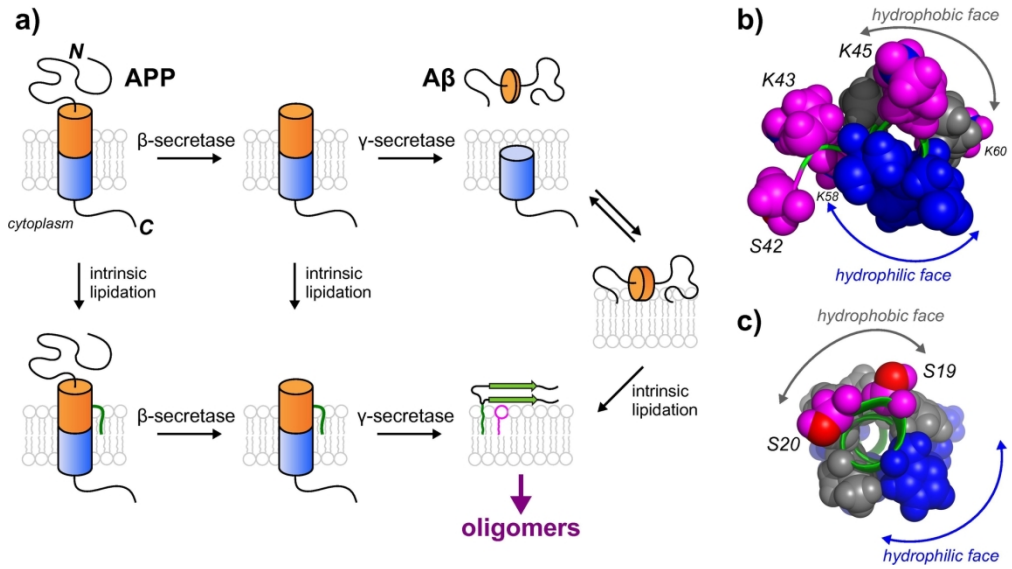
Lipidated propranolol species formed 24 h after administration of propranolol to Hep G2 cells. RC=O includes myristoyl, palmitoyl, palmitoleoyl, stearoyl and oleoyl, plus other minor products.

83x55mm (300 x 300 DPI)



Protein folding pathways leading to the formation of fibrils. Path a is the nucleation pathway in the absence of membranes; path b is an alternative pathway in or on the membrane; path c involves facilitated nucleation by a low energy lipidated peptide formed by intrinsic lipidation. a) Schematic representation of key intermediates. Most aggregation intermediates comprise an ensemble of microstates. b) Energy level diagram. Some intermediates have been omitted for clarity. Adapted by permission from Springer Nature: Nature, Molecular chaperones in protein folding and proteostasis, Hartl *et al*., 2011.[86]

170x78mm (300 x 300 DPI)



Proposed role of intrinsic lipidation in amyloid formation. a) Pathways for nucleation of Aβ peptides. Shown at the top, secretase cleavage of APP releases an Aβ peptide which undergoes reversible binding to the membrane in an orientation that favours acyl transfer to the peptide. An alternative route involving lipidation of APP before secretase cleavage is shown at the bottom. b) Residues 7 and 25 of the N-terminal section of α-syn (PDB entry 1XQ8 [103] viewed along the helix axis with residue 7 nearest the viewer. c) Residues 42–59 of hIAPP (PDB entry 2KB8), looking down the helix axis with residue 42 closest to the viewer. In b) and c), amphiphilic sequences from amyloid forming peptides. Lysine and serine residues are shown in magenta, hydrophilic residues in blue and hydrophobic residues in grey.

168x94mm (300 x 300 DPI)