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Differential destabilization of membranes by tryptophan and phenylalanine during freezing: the roles of lipid composition and membrane fusion

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Abstract

The stability of cellular membranes during dehydration can be strongly influenced by the partitioning of amphiphilic solutes from the aqueous phase into the membranes. The effects of partitioning on membrane stability depend in a complex manner on the structural properties of the amphiphiles and on membrane lipid composition. Here, we have investigated the effects of the amphiphilic aromatic amino acids Trp and Phe on membrane stability during freezing. Both amino acids were cryotoxic to isolated chloroplast thylakoid membranes and to large unilamellar liposomes, but Trp had a much stronger effect than Phe. In liposomes, both amino acids induced solute leakage and membrane fusion during freezing. The presence of the chloroplast galactolipids monogalactosyldiacylglycerol or digalactosyldiacylglycerol in egg phosphatidylcholine (EPC) membranes reduced leakage from liposomes during freezing in the presence of up to 5 mM Trp, as compared to membranes composed of pure EPC. The presence of the nonbilayer-forming lipid phosphatidylethanolamine increased leakage. Membrane fusion followed a similar trend, but was dramatically reduced when the anthracycline antibiotic daunomycin was incorporated into the membranes. Daunomycin has been shown to stabilize the bilayer phase of membranes in the presence of nonbilayer lipids and was therefore expected to reduce fusion. Surprisingly, this had only a small influence on leakage. Collectively, these data indicate that Trp and Phe induce solute leakage from liposomes during freezing by a mechanism that is largely independent of fusion events.

Keywords: Aromatic amino acid; Freezing; Galactolipid; Liposome; Nonbilayer lipid; Thylakoid membrane

1. Introduction

As an adaptive answer to environmental stress conditions such as drought and low temperature, higher plants accumulate different solutes, which are thought to have protective functions under stress conditions. A functional role for different sugars, the amino acid proline, quaternary ammonium compounds such as glycinebetaine, and some specific proteins in plant stress tolerance is now well established and many of these substances are able to stabilize membranes in the frozen or dry state (see [1–6] for reviews). More recently, the influence of small, amphiphilic molecules on membrane stability under stress conditions has been investigated. It was found that amphiphiles, that are mainly present in solution

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in fully hydrated cells, may partition into the cell membranes under conditions of dehydration [7]. The degree of partitioning is not only dependent on the sample water content, but also on the polarity of the solute [8]. This partitioning influences the phase state of the bilayer and its stability [7,9]. In addition, many amphiphilic substances such as flavonols or hydrochinones also have antioxidative properties and may therefore protect membranes from oxidative damage under conditions of low water availability (see [3,10] for reviews). There is good evidence that in desiccation tolerant cells, the partitioning of amphiphiles into membranes is a regulated process [8].

The direct effects of any given amphiphile on membrane stability, however, cannot be easily predicted. In a detailed investigation with the glycosylated hydrochinone arbutin, which has been found in high concentrations in extremely freezing or desiccation tolerant plant species [11,12], it was found that its effects on membrane stability depended entirely on the lipid composition of the target membrane. Arbutin was cryoprotective for isolated chloroplast thylakoid membranes, but severely damaged phosphatidylcholine vesicles during freezing or drying. Protection of model membranes by arbutin required the presence of a nonbilayer-forming lipid, either the chloroplast glycolipid monogalactosyldiacylglycerol (MGDG) or the phospholipid phosphatidylethanolamine (PE) [13,14]. It could be shown that arbutin specifically stabilized the bilayer phase of membranes containing nonbilayer lipids [14]. However, not all amphiphiles show this behavior [3]. Therefore, more knowledge is needed about the stabilizing or destabilizing effects of biologically relevant amphiphiles on membranes under stress conditions, in order to understand the role that such substances may play in cellular stress tolerance.

Amino acids are ubiquitously found in all living cells and the aromatic amino acids Tyr, Trp and Phe have an amphiphilic character due to the hydrophobic phenyl or indole rings. Therefore, although they are usually only present in low concentrations in cells, aromatic amino acids represent interesting model substances to further study the effects of relatively hydrophilic amphiphiles on membrane stability. Due to its exceedingly low solubility in water, no meaningful experiments could be carried out with Tyr and we have therefore limited this investigation to Phe and Trp. While there has been extensive interest in the functional and structural role of aromatic amino acids in proteins, especially in membrane proteins and membrane active peptides, there are almost no data available about the effects of the free amino acids on membrane stability. It has, however, been shown that Phe may damage spinach thylakoid membranes during freezing [15].

In the present contribution we have shown that both Trp and Phe were damaging to thylakoid membranes at very low concentrations during freezing. In liposomes, both amino acids induced solute leakage and membrane fusion under the same conditions. Trp had in general a much stronger effect than Phe and the action of both amino acids was modulated by the membrane lipid composition. Our results highlight the complexity of the effects that small amphiphiles can have at relatively low concentrations on membrane stability under stress conditions.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, AL), egg phosphatidylethanolamine (EPE) was from Sigma (St. Louis, MO). *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-Phosphatidylethanolamine (NBD-PE), *N*-(lissamine Rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine (Rh-PE) and carboxyfluorescein (CF) were obtained from Molecular Probes (Leiden, The Netherlands). CF was purified according to [16]. The chloroplast glycolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were purchased from Lipid Products (Redhill, Surrey, UK). Daunomycin was received from Sigma, Trp and Phe from Fluka (Deisenhofen, Germany).

2.2. Preparation of liposomes

Liposomes were composed (on a weight basis) of either 50% DGDG/50% EPC, 40% MGDG/60% EPC, 50% EPE/50% EPC or 100% EPC. MGDG and EPE are nonbilayer lipids and, therefore, liposomes are severely destabilized with higher concentrations in the membranes [14,17]. Liposomes for

leakage studies were made as previously described [17]. Briefly, 5 mg of lipid were hydrated in 250 μl of 100 mM CF, 10 mM TES and 0.1 mM EDTA (pH 7.4) and extruded using a Liposofast hand-held extruder ([18]; Avestin, Ottawa, Canada) with 100-nm pore filters. To remove external CF, the liposomes were passed over a Sephadex G-25 column (NAP-5, Pharmacia) in 10 mM TES, 0.1 mM EDTA and 50 mM NaCl (TEN buffer, pH 7.4). Liposomes for fusion assays were made with the same lipid compositions as for leakage, with the addition of 1 mol% each of the fluorescent probe pair NBD-PE and Rh-PE. Where indicated, the anthracycline daunomycin was mixed with lipids in organic solvent before formation of liposomes, in a lipid: daunomycin molar ratio of 20 [19].

2.3. Freezing of liposomes

Equal volumes of liposomes (10 mg lipid/ml) and concentrated solutions of amino acids in TEN were combined (40 μl/sample) to reach the final amino acid concentrations indicated in the figures. Samples were frozen rapidly in an ethylene glycol bath precooled to −20°C. After 3 h of incubation, samples were warmed quickly to room temperature in a water bath. Controls were incubated on ice for 3 h.

2.4. Leakage and fusion measurements

CF fluorescence is self-quenching when the dye is trapped inside the liposomes at high concentrations and fluorescence is increased when the dye is released into the medium. Leakage was determined as described previously [17] by measuring fluorescence at room temperature with a Kontron SFM 25 fluorometer (Bio-Tek Instruments, Neufahrn, Germany) at excitation and emission wavelengths of 460 and 550 nm, respectively.

Liposome fusion after freezing and thawing was determined using fluorescence resonance energy transfer [20] as described in detail in a recent publication [17]. Briefly, two liposome samples were prepared: one sample was labeled with both NBD-PE and Rh-PE, while the other sample was unlabeled. The two samples were combined after extrusion in a 1:9 (labeled/unlabeled) ratio, resulting in a final lipid concentration of 10 mg/ml. The liposomes were mixed with amino acid solutions in the same manner as for the leakage experiments. Fusion was measured by fluorescence resonance energy transfer [20] with a Kontron SFM 25 fluorometer at excitation and emission wavelengths of 450 and 530 nm, respectively.

Leakage and fusion values reported in the figures always represent mean ± S.D. from three parallel samples. Where no error bars are visible, they were smaller than the symbols.

2.5. Isolation and freezing of thylakoid membranes

Thylakoid membranes were isolated from the leaves of 14-day-old pea plants (*Pisum sativum* L.) as described in [21]. Isolated thylakoid membranes were washed three times and finally suspended in a simplified artificial stroma medium (10 mM $MgCl_2$, 20 mM K_2SO_4, 150 mM Na-glutamate and 50 mM sucrose [22]). Different concentrations of Trp or Phe, dissolved in the same solution, were added and samples, containing approximately 2 mg chlorophyll/ml, were frozen at −20°C. After 3 h, samples were rapidly thawed in a water bath at room temperature. Photochemical activity of Photosystem II (PS II) was determined polarographically as the rate of oxygen evolution with phenyl-*p*-benzoquinone (pBQ) as an electron acceptor at room temperature. The reaction medium contained 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 20 mM MES (pH 6.5), 0.1 mM pBQ and thylakoid membranes equivalent to 15 μg chlorophyll/ml. Chlorophyll concentration was determined according to Arnon [23].

3. Results

As a first step in our investigation of the effects of aromatic amino acids on membrane stability we used isolated chloroplast thylakoid membranes as a well-characterized model for freeze–thaw damage in a biological membrane (see [6] for a review). Thylakoid membranes were suspended in an artificial stroma medium [22], in which the same kind of freeze–thaw damage occurs that has also been found in thylakoids frozen and thawed in vivo [24]. Damage, determined as a reduction in the photochemical activity of PS II as a function of the concentrations of Phe or Trp, is shown in Fig. 1. PS II activity de-

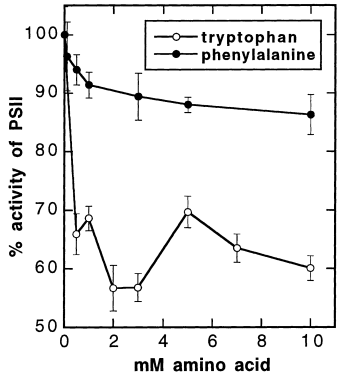

Fig. 1. Photochemical activity of Photosystem II (PS II) of thylakoid membranes after freezing for 3 h at −20°C in the presence of different concentrations of the aromatic amino acids Trp or Phe. 100% activity of PS II corresponds to 50.01 μmol O_2/mg chlorophyll × h. Mean values ± S.D. from four independent experiments are shown.

creased with increasing concentrations of both amino acids. However, the decrease was much stronger in the presence of Trp than in the presence of Phe, especially at low concentrations. At concentrations above approximately 2 mM, no significant further increase in damage was observed.

Thylakoid membranes are a complex biological system. In order to elucidate whether the cryotoxic effects we observed with the aromatic amino acids (Fig. 1) are related to a specific membrane component, we used liposomes for a further detailed analysis. The most abundant thylakoid lipids are the uncharged galactolipids MGDG and DGDG, comprising about 75% of the total lipid content. The remaining portion of the acyl lipids are made up by the anionic lipids sulfoquinovosyldiacylglycerol and phosphatidylglycerol. The anionic lipids and DGDG are bilayer-forming lipids, while MGDG is a nonbilayer lipid [25]. In this investigation, we used model membranes composed of four different types of lipids: the bilayer-forming lipids EPC or DGDG, and the nonbilayer lipids EPE or MGDG. Amino acids were added to the outside of the liposomes only, to make the results directly comparable to those reported in Fig. 1 and most data in the literature. In every experiment, parallel samples with or without 10 mM Trp or Phe were incubated for 3 h at 0°C. In no case did the presence of the amino acids increase the degree of leakage or fusion under non-freezing conditions.

Increasing concentrations of Trp caused massive leakage of CF from all types of liposomes during freezing (Fig. 2A). Significant differences between liposomes made from different lipids, however, were observed at low Trp concentrations. Here, the highest degree of release of the internal content after freezing was observed for liposomes containing 50% EPE, followed by liposomes made from pure EPC. Liposomes containing one of the galactolipids showed the least amount of damage. In fact, the presence of MGDG in the membranes led to a small, but highly reproducible, reduction in leakage at very low Trp concentrations (up to 1 mM). This indicates that the presence of sugars in the lipid headgroups

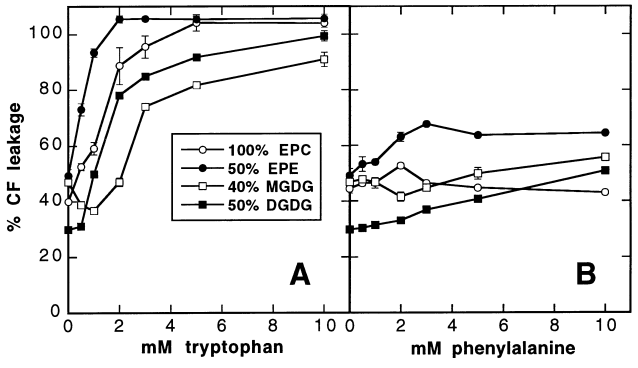

Fig. 2. Effect of Trp (A) and Phe (B) on freeze-induced damage to large unilamellar liposomes. Samples were frozen for 3 h at −20°C and damage was determined after thawing as leakage of the soluble fluorescent marker carboxyfluorescein (CF). Liposomes were prepared either from pure EPC or from mixtures of EPC with the indicated weight fractions of MGDG, EPE or DGDG.

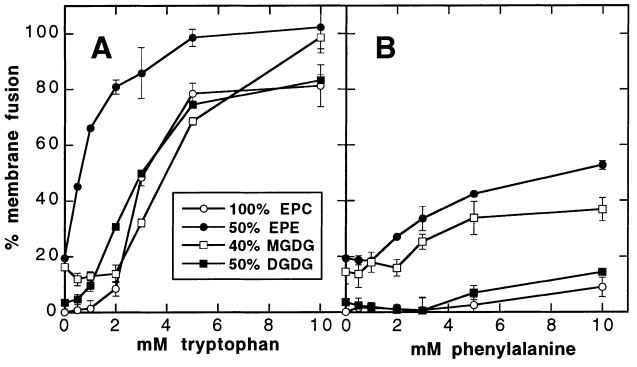

Fig. 3. Freeze–thaw damage to liposomes frozen in the presence of different concentrations of Trp (A) or Phe (B), determined as vesicle membrane fusion. Liposomes were either prepared from pure EPC or with the addition of different fractions of MGDG, EPE or DGDG as indicated.

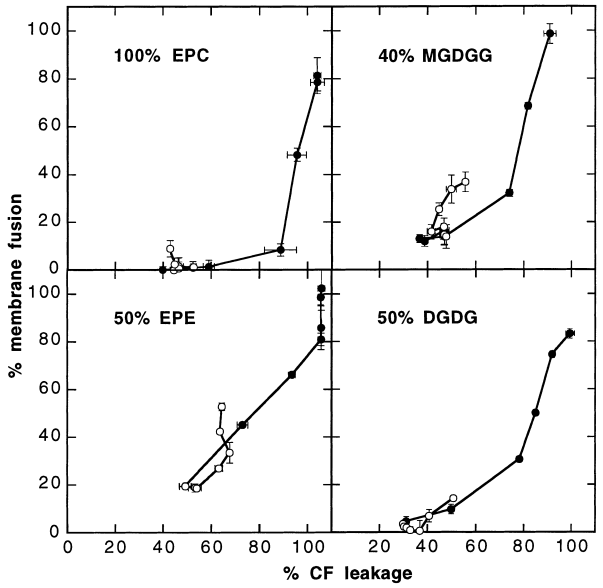

Fig. 4. Analysis of the correlations between CF leakage (cf. Fig. 2) and membrane fusion (cf. Fig. 3) for liposomes frozen in the presence of different concentrations of Trp (solid symbols) or Phe (open symbols). The membranes contained EPC and different weight fractions of the indicated lipids.

may have a stronger influence on the cryotoxicity of Trp than the phase preference of the lipids. Similar to our results with thylakoids (Fig. 1), the effect of Phe on freeze-induced leakage was much weaker (Fig. 2B). Only in the case of vesicles containing 50% EPE or 50% DGDG a small destabilization was observed with increasing concentrations of Phe.

During freezing pure water is removed from solution as ice and the concentrations of solutes and membranes in the residual unfrozen part of the system are dramatically increased. Under these conditions of partial dehydration and close proximity, membrane fusion may occur, which often leads to solute leakage. The presence of Trp in the suspending medium during freezing induced strong fusion of liposomes for all lipid compositions tested (Fig. 3A). As in the case of leakage, there were significant differences between different types of liposomes at low Trp concentrations. In liposomes containing 50% EPE, fusion was significantly increased over the whole concentration range. For the other three types of liposomes, fusion only started to increase at Trp concentration above 1–2 mM. As in the case of leakage, there was a strong difference between liposomes containing the nonbilayer lipids EPE or MGDG, again indicating a dominating influence of the sugars in glycolipid headgroups over the phase preference of the lipids. The effect of Phe was much weaker than that of Trp (Fig. 3B). In this case, however, liposomes containing the nonbilayer lipids were more affected than liposomes containing only bilayer lipids.

The data from the freezing experiments reported above were further analyzed in Fig. 4 by exploring possible correlations between leakage and fusion. As noted above, the effect of Phe was generally much weaker than that of Trp. For liposomes containing 50% EPE, there was a linear correlation between leakage and fusion for samples containing Trp, which only broke down for samples that had already suffered complete leakage. In the case of Phe, there was also a correlation, albeit not linear. Liposomes composed of pure EPC and those containing galactolipids, showed a much stronger increase in leakage than in fusion at low concentrations of Trp. At higher concentrations, where leakage had already reached more than 80%, there was a strong increase in fusion. For both amino acids, there was no correlation be-

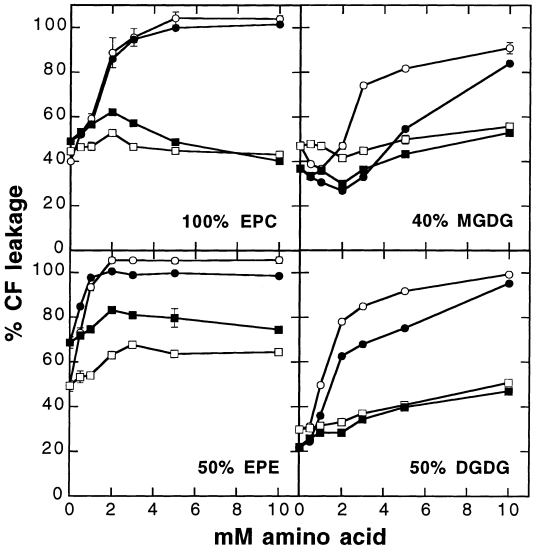

Fig. 5. Effects of the anthracycline antibiotic daunomycin on the freeze–thaw stability of liposomes in the presence of different concentrations of Phe (■, □) or Trp (●, ○). The membranes contained EPC and different weight fractions of the indicated lipids. In addition, samples indicated by solid symbols contained 5 mol% of daunomycin. Membrane stability during a freeze–thaw cycle was assessed as carboxyfluorescein (CF) leakage.

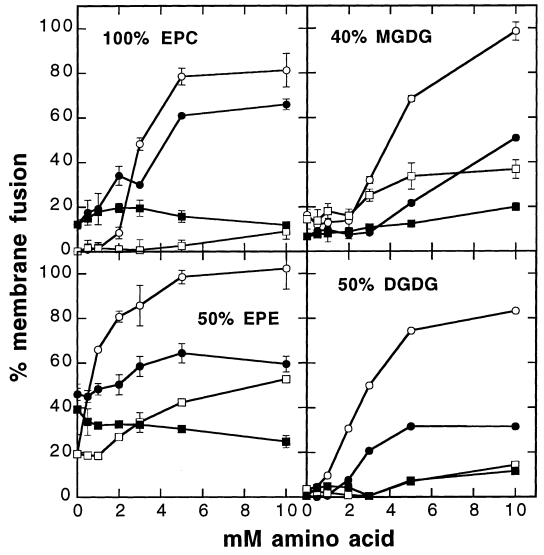

Fig. 6. Freeze–thaw damage to liposomes frozen in the presence of different concentrations of Trp or Phe, determined as vesicle membrane fusion. See the legend to Fig. 4 for details.

tween fusion and leakage for liposomes made from pure EPC, but a nonlinear correlation for liposomes containing 50% DGDG. For liposomes containing MGDG, there was again no simple linear correlation, but in this case the correlations were clearly different for Trp and Phe. These results indicate that both amino acids are to some extent fusogenic during freezing and that fusion may contribute to the observed leakage, at least in membranes that were not exclusively made from EPC.

It has been suggested that fusion proceeds through the formation of nonbilayer structures in membranes and that nonbilayer lipids increase the propensity of membranes to undergo fusion events [26,27]. In order to determine to what extent the formation of nonbilayer phases was responsible for the observed effect of freeze-induced destabilization of membranes in the presence of Trp and Phe, we used the anthracycline antibiotic daunomycin, which has been reported to partition into lipid bilayers [28] and to stabilize bilayer structures in membranes containing nonbilayer lipids [19].

Fig. 5 shows the influence of daunomycin on CF leakage from liposomes, frozen in the presence of Trp or Phe. Daunomycin itself increased the extent of leakage from liposomes frozen in buffer by 10–20% in the case of membranes containing only the phospholipids EPC and EPE, and reduced leakage by 10% for vesicles containing galactolipids. Unexpectedly, the suppression of the formation of a nonlamellar phase had little effect on the freeze-induced release of CF. A strong stabilization of the membranes was only observed in the case of liposomes containing 40% MGDG frozen in the presence of Trp. Quite remarkably, there was no effect on membranes containing 50% of the nonbilayer lipid EPE, but a clear effect at Trp concentrations up to 5 mM for membranes containing a mixture of the bilayer lipids EPC and DGDG. In the presence of Phe, the incorporation of daunomycin into the membranes had no clear effect on the freeze-induced leakage of the liposomes, regardless of lipid composition (Fig. 5).

The effect of daunomycin on freeze-induced fusion was much more pronounced (Fig. 6). In the absence of amino acids, the presence of daunomycin in the membranes resulted in a small increase in fusion for liposomes made only of phospholipids. This correlates well with the increase in leakage observed under the same conditions (Fig. 5). Also in agreement with the leakage data was the reduction in fusion observed with MGDG-containing liposomes in the absence of amino acids. The addition of Trp to the samples led to a much smaller increase

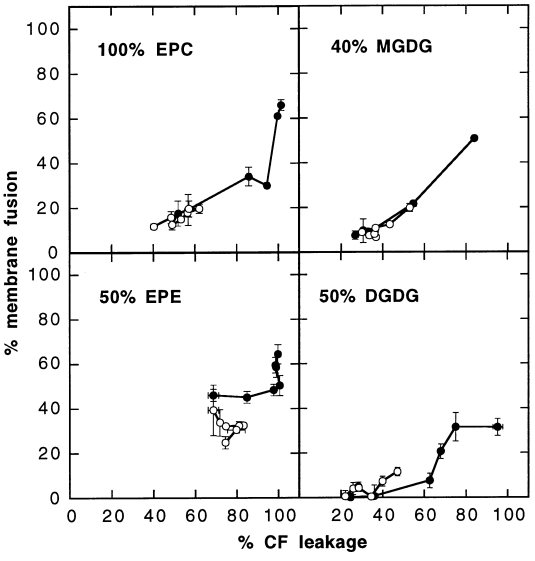

Fig. 7. Analysis of the correlations between CF leakage and membrane fusion for liposomes frozen in the presence of Trp (solid symbols) or Phe (open symbols). The membranes contained EPC, 5 mol% daunomycin, and different weight fractions of the indicated lipids.

in fusion for all membranes containing daunomycin compared to those not containing the drug. Most strikingly, for liposomes containing 50% EPE, fusion increased by 80%, from 20% in the absence of Trp to 100% at the highest Trp concentration, while for liposomes also containing daunomycin this increase was reduced to 17%, from 45% to 62%. A similar effect of daunomycin was observed for liposomes containing 40% MGDG or 50% DGDG. Liposomes formed from pure EPC showed a smaller, but nevertheless clear, effect of daunomycin on Trp-induced fusion during freezing, reducing the increase in fusion between 0 and 10 mM Trp from 81% to 54%. A decreased fusogenic effect in the presence of daunomycin was also observed for Phe with membranes containing the nonbilayer lipids MGDG or EPE. For MGDG containing vesicles, for instance, the reduction in fusion at the highest Phe concentration was 20%. If we consider that the maximal amount of fusion induced by Phe was only 40%, then the effect of daunomycin was a twofold reduction. For EPE containing liposomes and for liposomes made from pure EPC, daunomycin completely suppressed Phe-induced fusion. There was no detectable effect of daunomycin on fusion of liposomes containing 50% DGDG, frozen in the presence of Phe, but the degree of fusion was extremely low in all samples.

The fact that the presence of daunomycin in the membranes had a much stronger effect on fusion than on leakage led to a loss of most of the correlations observed between fusion and leakage for liposomes made without the drug (Fig. 7; compare Fig. 4). This was true, both for samples frozen in the presence of Trp and Phe. Only in the case of membranes containing 40% MGDG a strong correlation between fusion and leakage was observed with membranes containing daunomycin.

4. Discussion

During freezing of plant leaves, water crystallizes in the apoplastic spaces and the cells are severely dehydrated. Damage to membranes during freezing both in vivo and in vitro is generally considered to be the consequence of dehydration, rather than of low temperatures per se (see [4,6,24] for reviews). The extent of damage can be related both to the extent of osmotic water loss and to the presence of membrane stabilizing or destabilizing solutes. Amphiphilic substances may be particularly interesting in this regard, as they are freely soluble under well-hydrated conditions, but partition into the membrane lipid phase as water is removed, e.g., as ice, and thus only exert effects on membrane stability under conditions of at least partial dehydration. The degree of partitioning of any given amphiphile will be the result of a complex interplay of the degree of dehydration, solute polarity, and solution viscosity [8]. Trp and Phe are good examples for this behavior, as it is not possible to measure any partitioning of these amino acids into well-hydrated membranes ([29]; Popova and Hincha, unpublished), nor do they have any measurable influence on membrane stability under these conditions (data not shown). Nevertheless, both have strong effects on membrane stability during freezing and it can be assumed that their aromatic ring structures insert into membranes under conditions of low water availability. This question is currently under investigation in our laboratories.

A deduction of the possible positioning of aromatic amino acids after partitioning into a membrane can be made from several reports of the location of such residues in membrane spanning proteins and peptides. Statistical analyses of the distribution of amino acids in integral membrane proteins showed a strong preference for the lipid–water interface of membranes for both Trp and Phe [30,31]. Likewise, studies using Trp analogs [29,32] or Trp-containing peptides [33–38] showed a strong preference of Trp to localize at the membrane surface. We would therefore expect the free amino acids to also partition into the headgroup or glycerol backbone regions of membranes during freeze-induced dehydration, but not into the hydrophobic core.

This partitioning at the membrane surface during freezing, however, had dramatic effects on the stability of both biological and model membranes. The presence of Trp or Phe in the samples during freezing led to a suppression of PS II-mediated electron transport in isolated chloroplast thylakoids (Fig. 1), indicating the cryotoxicity of the amino acids for biological membranes. The photochemical activity of PS II

has been shown to be a sensitive indicator for the intactness of the photosynthetic electron transport chain in chloroplasts, both in vivo and in vitro. Damage occurs predominantly at the water-splitting site of PS II, presumably due to the loss of proteins from the internal (lumenal) side of the thylakoid membranes [39,40]. Such a loss of lumenal thylakoid proteins during freezing has been extensively documented for the electron transport protein plastocyanin [6,24]. In a liposome system, this freeze–thaw-induced leakage of proteins from thylakoids may be modeled as the leakage of the fluorescent dye CF from lipid vesicles [13].

For thylakoids, the detrimental effect of Phe was much smaller than that of Trp (Fig. 1). This difference between Trp and Phe also persisted in all experiments with liposomes, where Trp led to much more leakage and membrane fusion than Phe, irrespective of the membrane lipid composition.

It is also interesting to compare the effects of the aromatic amino acids with that of the amphiphile arbutin. In liposomes made only from EPC, complete leakage after freezing was achieved in the presence of 20 mM arbutin [13] or 6 mM Trp (Fig. 2). With Phe, complete leakage could not be induced within its solubility limits. The most striking differences between Trp and arbutin were manifest in the presence of liposomes containing the nonbilayer lipid EPE. The presence of increasing concentrations of EPE in EPC membranes reduced the leakage and fusion induced by arbutin in pure EPC liposomes [13]. In the presence of Trp, on the other hand, both leakage and fusion were strongly increased in membranes containing EPE, as compared to membranes made only from EPC (Figs. 2 and 3). As fusion is believed to proceed through nonbilayer intermediates [26,27], it could be expected that the presence of a nonbilayer lipid in the bilayer would increase vesicle fusion under stress conditions. However, in the absence of Trp, fusion only increased by 20% when liposomes containing pure EPC were compared with vesicles made from 50% EPE and 50% EPC. Dramatic differences between these two types of liposomes only became evident when low concentrations of Trp were added to the samples (Fig. 3). Since higher concentrations of Trp also induced fusion during freezing of liposomes made entirely of bilayer lipids (100% EPC or 50% DGDG and 50% EPC), it seems possible that the partitioning of Trp into membranes may facilitate the formation of fusion intermediates.

Trp elicited both leakage and fusion in liposomes containing 50% EPE. There was a linear correlation between leakage and fusion for samples containing low concentrations of Trp (Fig. 4). This seemed to indicate that leakage was a consequence of vesicle fusion events. In order to clarify, whether this correlation was really the result of a causal relationship, we incorporated the anthracycline antibiotic daunomycin into the membranes. Daunomycin has been shown to stabilize the bilayer phase in membranes containing PE [19]. The stabilization of bilayer structures should impair the formation of nonbilayer intermediates necessary for fusion [41]. This expectation was borne out by fusion measurements, which showed a strong reduction in vesicle fusion during freezing in the presence of Trp (Fig. 6). Unexpectedly, however, no concomitant reduction in leakage was detected (Fig. 5) and the previous correlation between fusion and leakage was no longer observed (Fig. 7). These data clearly indicate that Trp induced leakage in these liposomes mainly through a nonfusogenic mechanism. This was also evident in membranes composed only of EPC, where no correlation between fusion and leakage existed, either in the absence (Fig. 4) or the presence (Fig. 7) of daunomycin in the membranes.

In the absence of amino acids, daunomycin had small, but reproducible, effects on membrane stability during freezing. Both leakage and fusion increased in membranes containing only phospholipids and decreased in membranes containing EPC and a galactolipid. As the effect was clearly not related to the phase preference of the lipids, we cannot at present offer a convincing explanation for this observation.

Interestingly, in the presence of Trp liposomes containing the nonbilayer lipid MGDG in their membranes, behaved much more like vesicles containing the bilayer galactolipid DGDG, than like those containing the nonbilayer phospholipid EPE. This was true both for leakage (Fig. 2) and fusion (Fig. 3). At low concentrations, Trp actually stabilized liposomes containing 40% MGDG. This is similar to the effect of arbutin [13], but the fact that Trp could not stabilize membranes containing EPE

points to a different kind of interaction in the case of the amino acid. We propose that Trp participates in hydrophobic stacking interactions between its aromatic side chain and the sugar ring structures of the galactolipid headgroups. Such interactions have been convincingly demonstrated for Trp residues in the sugar-binding cavities of several lectins [42], for Trp in proteins interacting with oligomeric glycans [43], and for Trp residues in bateriorhodopsin interacting with the sugar moieties of the galactolipids of the purple membrane [44].

In contrast to Trp, Phe induced leakage or fusion only in liposomes containing a nonbilayer lipid (Figs. 2 and 3). There were no significant differences between membranes containing either EPE or MGDG. The addition of daunomycin to these membranes completely abolished freeze-induced damage to the liposomes in the presence of Phe. Although there were no clear correlations between leakage and fusion under these conditions (Figs. 4 and 7), in part probably due to the low total amount of damage elicited by Phe, we conclude from these data that Phe induced leakage during freezing predominantly in nonbilayer lipid-containing membranes through a fusogenic mechanism.

In conclusion, the data presented in this paper represent a further step in our efforts to understand the molecular mechanisms of plant membrane damage and protection under stress conditions. It is apparent that the effects of amphiphiles on membrane stability depend in a highly complex manner on the chemical structure of the solutes and the lipid composition of the membranes. Since only the effects of three amphiphilic substances (arbutin, Trp, Phe) have been analyzed in any detail, no predictions based on molecular structure of the effects of such solutes on membrane stability under stress conditions can be made at present. It can be assumed that other constituents of biological membranes, such as proteins, also play a role in determining the effects of solutes on membrane stability during freezing or drying. This is exemplified by the fact that low concentrations of Trp already strongly damaged thylakoid membranes during freezing (Fig. 1), but had no effect on leakage or fusion in liposomes containing the predominant thylakoid lipids MGDG or DGDG (Figs. 2 and 3).

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