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Mechanisms of interaction of amino acids with phospholipid bilayers during freezing

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In this study we compare the ability of various amino acids to protect small unilamellar vesicles against damage during freeze/thaw. Liposomes were composed of 75% palmitoyloleoyl phosphatidylcholine and 25% phosphatidylserine. Damage to liposomes frozen in liquid nitrogen and thawed at 20°C was assessed by resonance energy transfer. Cryoprotection by numerous amino acids was compared in the presence and absence of 350 mM NaCl. The majority of amino acids with hydrocarbon side chains increased membrane damage during freeze/thaw regardless of the presence of salt. However, amino acids with hydrocarbon side chains of less than three carbons long, e.g. glycine, alanine, and 2-aminobutyric acid, were cryoprotective only in the presence of salt. We suggest that NaCl selectively increases the solubility of such amino acids, allowing them to act as cryoprotectants. In contrast, amino acids with side chains containing charged amine groups were cryoprotective regardless of the presence of salt. The degree of charge on the second amine group is shown to be important for cryoprotection by these molecules. We present evidence that suggests an interaction between the positively charged, second amine group of the amino acid, and the negatively charged phospholipid headgroup.

Introduction

A variety of organisms are known to accumulate amino acids in response to cold temperatures [1,2]. These amino acids are thought to play a role in preventing damage to cell structure during freeze stress. It has recently been shown that cold resistance by the mussel *Mytilus edulis* might be accomplished by the accumulation of amino acids within the blood [3]. Furthermore, cryoprotection by these compounds was shown to depend on plasma levels of salt (unpublished observations). The present study was conducted to determine the effectiveness of amino acids as membrane cryoprotectants in the presence and absence of plasma levels of salt. By investigating the role of salt, we have gained insight into the mechanisms of cryoprotection employed by various amino acids.

Previous studies have shown that certain combinations of salts and amino acids are able to minimize damage to isolated thylakoid mem-

Abbreviations: RET, resonance energy transfer; Tes, 2-{[2-hydroxy-1, 1-bis-(hydroxymethyl)ethyl]amino}ethanesulfonic acid. SUV, small unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; PS, phosphatidylserine.

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branes that were cooled to −25°C [4,5]. The authors suggested that the solutes protected by a colligative mechanism, increasing the amount of unfrozen water at −25°C. We provide an alternative explanation here. In the present study, small unilamellar vesicles were used as a model membrane system in which damage incurred during freeze/thaw could be monitored by resonance energy transfer (RET) [7]. Sodium chloride (350 mM) was used to mimic the blood osmolarity of a typical marine invertebrate. Phospholipid membranes were frozen in liquid nitrogen (−196°C) for 3 min, ruling out any protective mechanism invoking unfrozen water. The effectiveness of amino acids as cryoprotectants was based on their ability to reduce lipid mixing, as measured by RET. We do not maintain that probe intermixing is necessarily due to true fusion, although fusion would result in such lipid mixing.

The results show that membrane damage is slightly increased by the presence of NaCl alone. However, some amino acids that drastically increased membrane damage during freeze/thaw, were transformed into cryoprotectants when combined with 350 mM NaCl. The majority of amino acids, whether damaging or cryoprotective, were relatively unaffected by the presence of salt. We believe that these results cannot be explained by the colligative properties of amino acid-salt solutions.

In addition, we suggest that amino acids containing positively charged amine groups in their sidechain interact directly with membrane phospholipids. We provide evidence to show that the degree of positive charge on the second amine group is critical for its interaction with negatively charged phospholipid headgroups, and that this interaction is critical for cryopreservation.

Materials and methods

Chemicals. Alanine, glycine, ornithine hydrochloride, valine, isoleucine, leucine, histidine, norvaline, aspartic acid, glutamine, 3-methyl histidine and 1-methyl histidine were purchased from Sigma (St. Louis, MO); arginine, lysine, glutamic acid, methylglutamic acid hemihydrate, 2-aminobutyric acid, 5-aminovaleric acid, 2,3-diaminopropionic acid monohydrochloride, 2,4-diaminobutyric acid dihydrochloride and europium nitrate pentahydrate were acquired from Aldrich (Milwaukee, WI); asparagine was obtained from Calbiochem (San Diego, CA). All solutions were made up in 10 mM Tes buffer with 0.1 mM EDTA and their pH was adjusted to 7 (at 20°C) with NaOH or HCl. Salt concentrations were adjusted for solutions of ornithine, diaminoproprionic acid, and diaminobutyric acid to account for the crystalline hydrochloride. All lipids used in this study were purchased from Avanti Polar Lipids (Pelham, AL) and were kept in stock solutions at −20°C. The donor probe, cholesterol anthracene-9-carboxylate, was obtained from Molecular Probes Inc. (Junction City, OR) and the acceptor, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine was from Avanti.

Preparation of vesicles. Small unilamellar vesicles (SUV) of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and phosphatidylserine (PS) in a 3 : 1 ratio (w/w) were prepared containing either a donor or an acceptor probe as described previously [6,7]. This POPC/PS ratio prevented probe intermixing before and after each freeze/thaw event. Lipids (10 mg/ml) were sonicated to clarity in 10 mM Tes (pH 7 at 20°C) containing 0.1 mM EDTA. Samples were prepared by combining equal volumes (12 μl) of donor and acceptor liposomes (10 mg/ml) with 176 μl of cryoprotectant. Samples were frozen in liquid nitrogen and thawed at room temperature. This freeze-thaw cycle was repeated three times for each sample.

Resonance energy transfer. Membrane mixing was measured by RET as in similar studies [6,8]. Since leakage of internal contents are known to occur when phospholipid membranes undergo a phase transition [9], i.e., during cooling, RET was used to investigate specifically membrane damage due to freeze/thaw. Membrane mixing, e.g., fusion, allows the donor and acceptor probes, trapped in separate vesicles, to intermix. This intermixing allows the transfer of resonance energy from donor to acceptor and is seen as a reduction in donor emission. The extent of probe intermixing can be quantified by exciting at 365 nm and reading the donor emission at 460 nm on a Perkin-Elmer LS-5 fluorescence spectrophotometer. Probe intermixing is expressed as the difference between the donor emission of unfrozen

samples (0% mixing) and that of liposomes prepared containing both donor and acceptor probes (100% mixing) as previously described [6]. Each datum point in the figures is the mean of three samples and the error bars denote 1 S.D.

The europium experiments involved the use of liposomes composed of 90% dimyristoylphosphatidylcholine and 10% phosphatidylserine. Europium nitrate pentahydrate was used in a 3:10 mole ratio (Eu^{3+} phospholipid) according to the original protocol [8].

Results and discussion

The results presented here suggest that direct interaction of amino acids with phospholipid bilayers is responsible for their cryoprotective effect. We will show that this interaction differs markedly, depending on the structure of the amino acid. The results and their interpretation are organized around such structures in the following discussion.

Amino acids with hydrocarbon sidechains

Amino acids with hydrocarbon sidechains (see Table 1) drastically increased membrane fusion in the absence of salt. For example, glycine increased probe intermixing from 30% to 80% in the absence of NaCl, but reduced fusion to nearly 10% in its presence (Fig. 1). The presence of salt had the most dramatic effect on those amino acids with sidechains shorter than three carbons (Fig. 2). An increase in hydrocarbon sidechain length from two carbons to three, i.e., 2-aminobutyric acid to valine, eliminates cryoprotection. On the other hand, the addition of a methyl group to glycine, i.e., alanine, enhances membrane stabilization (Fig. 2). It is surprising that this effect is so specific, but we believe an explanation already exists in the physical chemistry literature, as discussed below.

The ability of inorganic salts to improve cryoprotection by certain amino acids has been reported previously [4,5,10]. Heber et al. [5] tested the ability of amino acids to protect against inactivation of thylakoid membranes cooled to −25°C. They reported that glycine and alanine were protective only in the presence of NaCl, whereas valine, leucine and isoleucine did not prevent inactivation in the presence or absence of salt, similar to the results reported here. In their study, thylakoid membranes were cooled to −25°C and held for 4 h before being warmed in a water bath at room temperature. They suggest that cryoprotection is afforded by the fraction of unfrozen water at the freezing temperature (−25°C). The amount of unfrozen water is due to a colligative effect that is dictated by the combined concentration of the different solutes. Heber et al. [5,11] suggest that amino acids and salts are both membrane-toxic, but have different sites of

TABLE I

CHEMICAL STRUCTURES OF AMINO ACIDS WITH HYDROCARBON SIDECHAINS

GLYCINE	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}H$
ALANINE	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}CH_3$
2-AMINOBUTYRIC ACID	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_3$
VALINE	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{-}CH_3$
NORVALINE	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_2\text{-}CH_3$
LEUCINE	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}CH_2\text{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{-}CH_3$
ISOLEUCINE	$^{-}OOC\text{-}\underset{\displaystyle {}^{+}NH_3}{\underset{|}{CH}}\text{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_3$

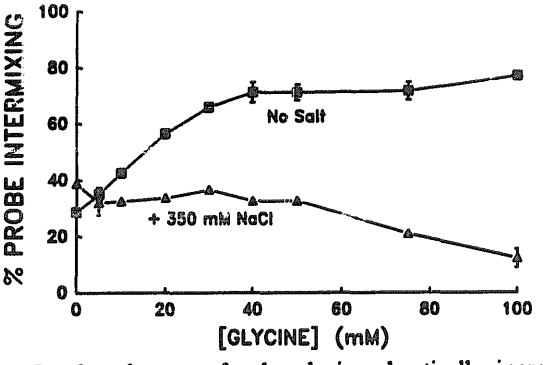


Fig. 1. In the absence of salt, glycine drastically increases membrane damage during freeze/thaw. However, when combined with 350 mM NaCl, glycine is cryoprotective.

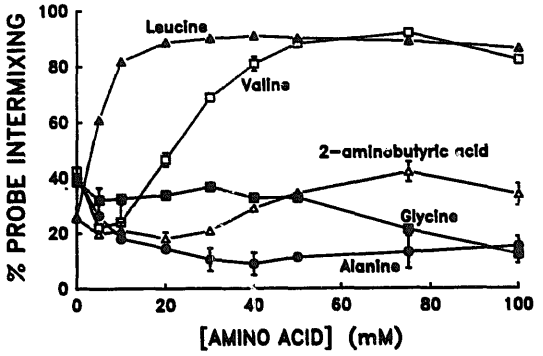


Fig. 2. In the absence of salt, all of these amino acids increase membrane damage similar to glycine, as seen in Fig. 1. When combined with 350 mM NaCl, amino acids with short hydrocarbon sidechains, e.g., glycine, alanine, 2-aminobutyric acid, are cryoprotective. Amino acids containing longer hydrocarbon sidechains, e.g., leucine and valine, are relatively unaffected by the presence of salt. Cryoprotection by norvaline and isoleucine followed a similar trend to that by leucine. See Table I for the chemical structures of these compounds.

toxicity. By using a combination of NaCl and amino acids, it is thought that specific membrane toxicity of individual components is minimized, while colligative action maintains a protective level of unfrozen water [4,5,10–12].

More recent studies by Santarius have detailed the effects of various salts on thylakoid membrane inactivation during cooling to −15°C [13]. He suggests that the thylakoid membranes remain in the unfrozen fraction, and are therefore subjected to high concentrations of solutes. Exposure of thylakoid membranes to the specific toxic effects of individual solutes is considered the source of inactivation [13]. It should be pointed out that many proteins are inactivated by low temperatures, and that the mechanism of protein destabilization or protection by solutes under such conditions has recently been elucidated [14]. We believe that many of the effects of specific salts on thylakoid membrane inactivation can be explained by this same mechanism, which is also known to account for solute-induced effects on protein stability in solution [14–16].

In the present study, phospholipid membranes were frozen in liquid nitrogen, eliminating unfrozen water as a vehicle for cryoprotection. We believe that increased membrane damage in the presence of amino acids might be due to eutectic crystallization during freezing, as demonstrated by Santarius [17]. Although cryoprotection by a number of amino acids was similar to that seen by Heber et al. [5], we offer a different explanation for the effect. We suggest that salts may serve to increase the solubility of certain amino acids, reducing membrane damage due to crystallization. Evidence for this explanation from other studies is discussed below.

While investigating freeze tolerance in *Mytilus edulis*, Williams [18] noted that "in the temperature range where animal survival is possible, taurine, and presumably the other amino acids, are able to stay in solution well below their eutectic temperatures". This effect on taurine solubility was only seen when an artificial intracellular solution containing 79 mM NaCl was used as the freezing medium [18]. Early solution studies on the effect of salt on amino acid solubility also demonstrate this salting-in effect [19,20]. Furthermore, increased amino acid solubility in the presence of salts is known to depend directly on the length of the amino acid hydrocarbon sidechain [20,21]. More specifically, the salting-in effect of NaCl is known to reverse with amino acid sidechains longer than three carbons [19–21]. In other words, the effect of NaCl is to increase the solubility of glycine and alanine, while decreasing the solubility of valine, leucine and isoleucine. This salting-in/salting-out effect by NaCl might well explain the results in Fig. 2. We are not suggesting that glycine, alanine and 2-aminobutyric acid are still in solution at liquid nitrogen temperatures, but we do suggest that the increased solubility of these compounds in the presence of NaCl plays a role in cryoprotection.

Amino acids with more complex sidechains

A similar salt effect was seen in cryoprotection by glutamine and asparagine (Fig. 3). Both of these compounds were damaging in the absence of salt, but were protective when combined with 350 mM NaCl (Fig. 3). Cryoprotection by the corresponding acidic amino acids, glutamic acid and aspartic acid, also depended on the presence of salt. Furthermore, the addition of a methyl group to glutamic acid's α carbon, i.e., methylglutamic acid, markedly increased cryoprotection by this compound (Fig. 4), similar to the effect seen when glycine was compared to alanine (Fig. 2). Al-

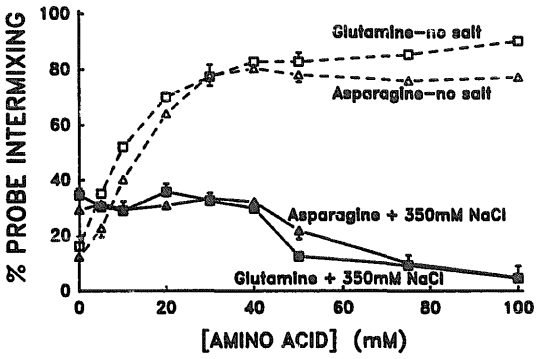


Fig. 3. The effect of salt on cryoprotection by glutamine and asparagine. Both of these amino acids have amide groups in their sidechains.

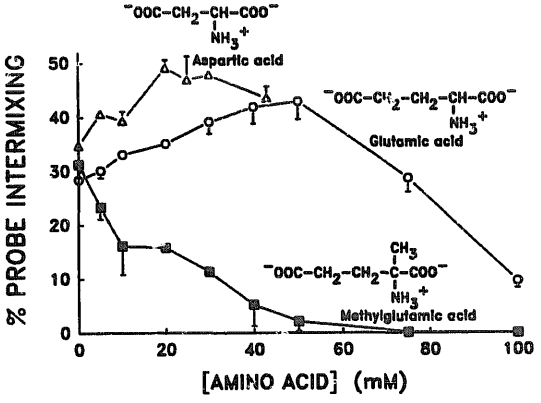


Fig. 4. Cryoprotection by acidic amino acids in the presence of salt. Note that the addition of a methyl group to the α carbon of glutamic acid greatly enhances cryoprotection. These compounds are only protective when combined with salt.

though the sidechains of these amino acids are not simply hydrocarbon, the results suggest that these amino acids have a protective mechanism similar to glycine, alanine and 2-aminobutyric acid.

TABLE II

CHEMICAL STRUCTURES OF BASIC AMINO ACIDS CONTAINING CHARGED AMINE GROUPS IN THEIR SIDECHAINS

ORNITHINE	$^{-}OOC\text{-}\underset{+NH_3}{\underset{\vert}{CH}}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$
LYSINE	$^{-}OOC\text{-}\underset{+NH_3}{\underset{\vert}{CH}}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$
ARGININE	$^{-}OOC\text{-}\underset{+NH_3}{\underset{\vert}{CH}}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}\overset{+NH_2}{\overset{\vert}{C}}\text{-}NH_2$
HISTIDINE	$^{-}OOC\text{-}\underset{+NH_3}{\underset{\vert}{CH}}\text{-}CH_2\text{-}C{=}CH$ (HN, NH, +C, H)

Amino acids with basic sidechains

We also investigated cryoprotection by other amino acids containing charged sidechains, e.g., lysine, arginine, histidine and ornithine, each of which has a positively charged amine group in their sidechain (Table II). In contrast to the amino acids mentioned earlier, all of these compounds were protective, regardless of the presence of salt (Fig. 5; we were unable to test the effectiveness of ornithine in the absence of salt because this compound is only available as a hydrochloride salt). The importance of the sidechain amine group in cryoprotection is demonstrated in Fig. 6. Ornithine, an effective cryoprotectant, is trans-

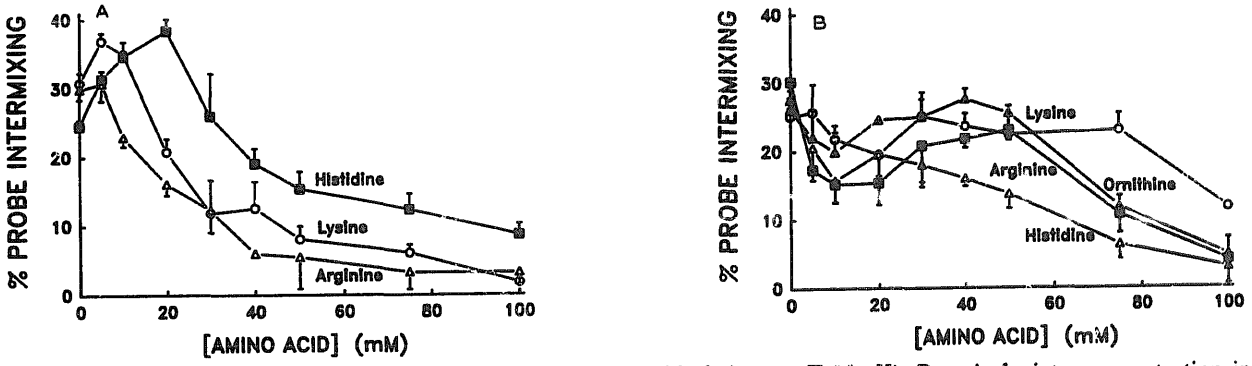


Fig. 5. Each of these amino acids has a second amine group in its sidechain (see Table II). Part A depicts cryoprotection in the absence of salt and part B depicts cryoprotection in the presence of 350 mM NaCl. Membrane protection by these compounds is not dependent on salt.

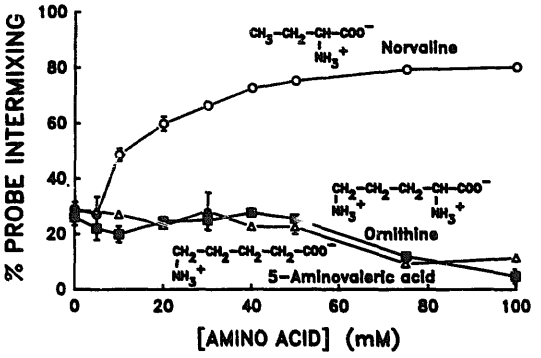


Fig. 6. Removal of ornithine's sidechain amino group destroys its ability to cryoprotect. In contrast, removal of ornithine's α amino group has almost no effect on cryoprotection. These data depict cryoprotection in the presence of 350 mM NaCl.

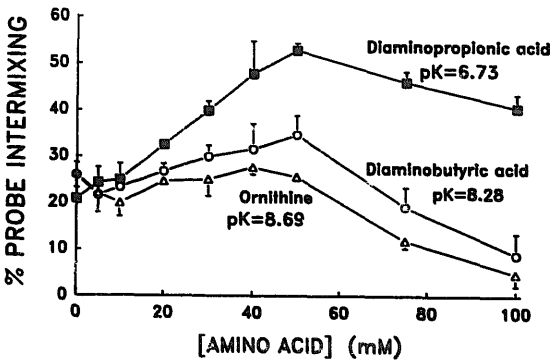


Fig. 7. The degree of charge on the second amine group is important for cryoprotection. Membrane protection by these compounds corresponds to the degree of charge on the sidechain amine group. Experiments were conducted at pH = 7.0 and the chemical structures are shown in Table III.

formed to a membrane-damaging compound by removal of its second amino group, i.e., norvaline. In contrast, when only the α amino group was removed, i.e., 5-aminovaleric acid, no decrease in cryoprotection was observed (Fig. 6). These results clearly indicate that the sidechain amine group is critical for cryoprotection of phospholipid membranes by this class of molecules.

Effect of charge on the sidechain

The sidechain amine groups of the compounds in Fig. 6 each carry a positive charge. It is possible that the degree of positive charge on the second amine group might be important for cryoprotection. To test this hypothesis, we compared cryoprotection by similar compounds (Table III) having different degrees of positive charge on the sidechain amine group. Fig. 7 shows that cryoprotection increases with an increase in positive charge on the second amine group. Diaminopropionic acid ($pK = 6.73$) was markedly less effective than either diaminobutyric acid ($pK = 8.28$) or ornithine ($pK = 8.69$) when frozen at pH = 7. Using the Henderson-Hasselbalch equation, the percent of amino groups carrying a positive charge at pH = 7 can be calculated. The calculations show that the second amino group of diaminopropionic acid is only 35% charged, whereas those of diaminobutyric acid and ornithine are 95% and 98% charged, respectively. We therefore suggest that the positive charge plays an important role in cryoprotection by these molecules.

This hypothesis is supported by the fact that cryoprotection by glutamine and asparagine depends on the presence of salt. Although these amino acids have a nitrogen in their sidechain, the nitrogen is part of an amide group and therefore cannot carry a positive charge. The absence of a positive charge on their sidechain nitrogen results in the inability of glutamine and asparagine to cryoprotect by the salt-independent mechanism of basic amino acids (Fig. 5). Instead, cryoprotection by glutamine and asparagine is similar to that seen with glycine and alanine (Figs. 1 and 2), suggesting a common cryoprotective mechanism among these four amino acids.

TABLE III

CHEMICAL STRUCTURES OF THREE STRUCTURALLY SIMILAR AMINO ACIDS

DIAMINOPROPIONIC ACID	$^{-}OOC\text{-}\underset{\overset{+}{N}H_3}{\underset{\vert}{C}}H\text{-}CH_2\text{-}NH_3^{+}$
DIAMINOBUTYRIC ACID	$^{-}OOC\text{-}\underset{\overset{+}{N}H_3}{\underset{\vert}{C}}H\text{-}CH_2\text{-}CH_2\text{-}NH_3^{+}$
ORNITHINE	$^{-}OOC\text{-}\underset{\overset{+}{N}H_3}{\underset{\vert}{C}}H\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_3^{+}$

Competition between positively charged amino acids and europium

The significance of the positive charge on sidechain nitrogens is not clear, based on the data

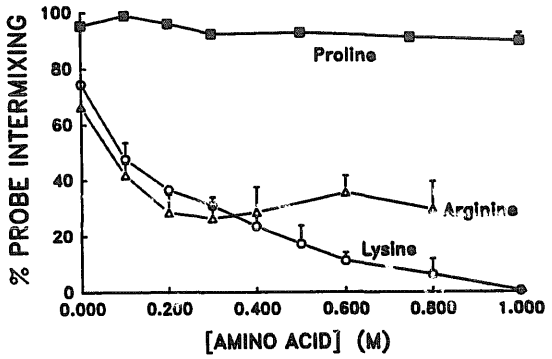


Fig. 8. Probe intermixing is greatly enhanced by the binding of europium to phospholipid headgroups. Lysine and arginine reduce membrane damage by europium, indicating an interaction between these amino acids and the phospholipid headgroup. Proline, a cryoprotectant that does not interact with the headgroup, is shown for comparison.

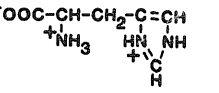
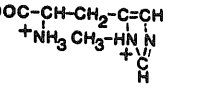
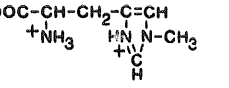
presented so far, but an ionic interaction between the sidechain amine group of the amino acid and the phosphate of membrane phospholipids is an interesting possibility for which we now provide the first evidence. According to our hypothesis, the sidechain amine group must be positively charged in order to interact with negatively charged sites on the bilayer. To investigate this interaction, we used the europium fusion assay as previously described [8]. This assay utilizes europium, a trivalent cation that binds to anionic sites on phospholipid headgroups [22], to induce fusion of vesicles subjected to freeze/thaw. Cryoprotective compounds that compete with europium for the phospholipid headgroup are known to reduce this fusion [8,23]. We tested the ability of arginine and lysine to reduce europium-induced fusion during freeze/thaw. The results in Fig. 8 show that these amino acids reduce fusion by europium, similar to other compounds that are known to bind to the phospholipid headgroup [8]. Results with proline, a cryoprotectant that does not interact with phospholipid headgroups [8], are shown for comparison. We suggest that these data are consistent with the possibility of an ionic interaction between the amino acid and the phospholipid headgroup.

Further evidence for ionic interaction between amino acids and phospholipids

Based on the observation that cryoprotection by histidine is observed only when PS is present in the membrane (data not shown), we suspected that the positively charged nitrogen of histidine's imidazole ring might interact directly with negatively charged phospholipids. To test this hypothesis, we compared the ability of 1-methyl histidine, 3-methyl histidine and histidine (Table IV) to reduce membrane damage during freeze/thaw. The substitution of a methyl group for a hydrogen on the positively charged nitrogen of the imidazole ring would be expected to hinder its interaction. The results in Fig. 9 are consistent with this hypothesis. Addition of a methyl group to the charged nitrogen in histidine's imidazole ring, i.e., 1-methyl histidine, resulted in reduced cryoprotection. In contrast, addition of a methyl group to the uncharged nitrogen of histidine, i.e., 3-methyl histidine, had no effect on cryoprotection. These

TABLE IV

CHEMICAL STRUCTURES OF THREE HISTIDINES

HISTIDINE	
1-METHYL HISTIDINE	
3-METHYL HISTIDINE	

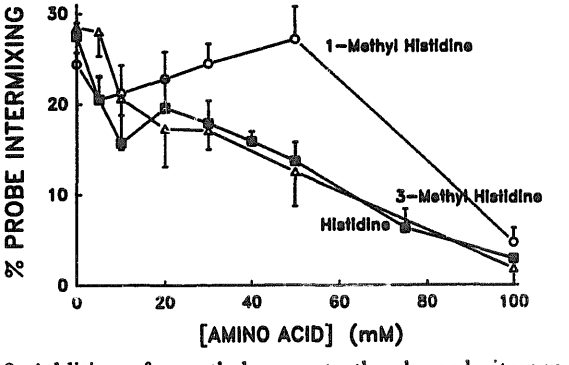


Fig. 9. Addition of a methyl group to the charged nitrogen of histidine's imidazole ring decreases its effectiveness as a cryoprotectant. In contrast, addition of a methyl group to the uncharged nitrogen of histidine has no effect on cryoprotection. These data depict cryoprotection in the presence of 350 mM NaCl. Chemical structures are shown in Table IV.

results are consistent with the hypothesis that the positively charged nitrogen of histidine's imidazole ring interacts directly with membrane phospholipids. However, there are remaining questions still to be resolved about the nature of this interaction. We should point out that free imidazole was not cryoprotective either in the presence or absence of salt (data not shown), indicating that the carboxyl and α amino groups may be important for cryoprotection in ways that are not yet clear.

Conclusions

We suggest that the dramatic effect of NaCl on cryoprotection by certain amino acids may be due to salting-in, allowing amino acids with short hydrocarbon chains to stay in solution at low temperatures. The mechanism by which these amino acids impart cryoprotection is still unclear. The presence of salts in the freezing medium could also neutralize charged groups on membranes and cryoprotectants, allowing interactions that would be unstable in the absence of salt due to charge repulsion. Whatever the mechanism, it is clear that salts can play an important role in membrane cryopreservation.

Cryoprotection by amino acids containing charged amine groups in their sidechains appears to be accomplished by a distinctly different mechanism. We suggest that basic amino acids minimize membrane mixing by interacting directly with membrane phospholipids, and sterically preventing close apposition of two bilayers. We suggest that this interaction is ionic in nature, and that the charge on the sidechain amine group is critical for cryoprotection by these molecules.

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