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THE EFFECT OF AMINO ACIDS ON THYLAKOID MEMBRANES DURING FREEZING AS INFLUENCED BY SIDE CHAIN AND POSITION ON THE AMINO GROUP

LINA TYANKOVA*

Botanisches Institut der Universität Düsseldorf, Düsseldorf (Germany)

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SUMMARY

Photophosphorylation of washed thylakoid membranes is inactivated during freezing for several hours at −25 °C. Amino acids with an amino group attached to a carbon atom in the β or a higher position of the carbon chain effectively protected the membranes against freezing damage in the absence of, and less so in the presence of, an inorganic salt such as NaCl.

α-Amino acids, on the other hand, were ineffective in the absence of but protective in the presence of NaCl. The extent of protection was a function of the amino acid/salt ratio and of the length of the carbon side chain. In balanced salt solutions glycine, α-alanine and α-aminobutyric acid were found to be protective, while α-amino caproic acid was incapable of exerting protection. Protection was increased by peptide formation, but still depended on the nature of the side chains.

INTRODUCTION

Thylakoid or mitochondrial membranes are useful as model systems to study harmful effects of freezing on cells or organisms by processes such as photophosphorylation and oxidative phosphorylation, both vital properties of intact cells, and both inactivated by freezing *in vitro* and *in vivo*[1]. With thylakoids, inactivation which is enhanced by a number of compounds, for instance inorganic salts, can be prevented by the presence of sufficient amounts of other compounds which are chemically as unrelated as sorbitol[2,3], dimethylsulfoxide[4], some amino acids[5,6], oligosaccharides[1,2], Heber's protein Factors I and II[7], and others. In view of the diversity of compounds capable of preventing inactivation it appears doubtful that a common mechanism underlies all protection phenomena. The effects of amino acids on thylakoids during freezing to −25 °C were particularly complex in that some amino acids such as proline or threonine protected very well in the absence of inorganic salts and less so in their presence, while others such as α-alanine or serine protected thylakoids only if inorganic salts were also present in a certain ratio to the amino acids[5,6]. Still other amino acids, for instance valine or phenylalanine, provided no significant protection with or without

* Present address: Doz. Lina Tyankova, Institute of Genetics and Plant Breeding, Academy of Agricultural Sciences, Sofia 13, Bulgaria.

salts present and rather abolished protection by other cryoprotectants[6]. This different behaviour was mainly attributed to differences in membrane toxicity of individual amino acids, even though the participation of eutectic phenomena could not be ruled out. In accordance with the proposals of Lovelock[8], protection by amino acids could be explained in part by their colligative properties which permitted them to reduce unspecifically the concentration of toxic components in the system at any one freezing temperature. However, individual interactions of amino acids with the membranes appeared to contribute also to protection. It was not clear to what extent the molecular structure and in particular the position of the amino group affected specific interactions. The experiments to be reported are intended to throw light on the latter problem.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves in a NaCl containing buffer[1,10] and washed with water to rupture the chloroplasts and reduce the NaCl concentration in the resulting thylakoid suspension to below $5 \cdot 10^{-3}$ M. The buffers, necessary for isolation of thylakoids, as well as the solutions of amino acids for the thylakoids treatment were adjusted to pH 7.8. In all cases the pH adjustment was accomplished with diluted HCl or NaOH. Equal volumes of a thylakoid suspension and of a solution of amino acid and/or NaCl were mixed and after 15 min preincubation at 0 °C frozen for 4 h at −25 °C. After thawing in a water bath, cyclic photophosphorylation was measured with phenazine methosulfate as a cofactor[1,10]. For controls thylakoids were used which were kept for 4 h at 0 °C with or without amino acids and/or NaCl also present. The photophosphorylation activity of the controls ranged between 400 and 600 μmoles/mg chlorophyll per h. Data given in any one figure were obtained in one single experiment, except those from Figs 2, 3 and 4.

Amino acids were obtained from Fluka, Buchs SG and Merck, Darmstadt.

RESULTS

Fig. 1 shows the effect peptide formation may have on the protection of thylakoids against freezing. Glycine alone was incapable of preventing inactivation of photophosphorylation by freezing but rather increased the damage. In proper combination with NaCl protection was observed. However, glycylglycine was protective even without NaCl. With NaCl also present, protection was decreased at low glycylglycine concentrations, but significantly increased at higher concentrations. At ratios of glycylglycine to NaCl higher than unity, protection of photophosphorylation against freezing was practically complete.

Protection as a function of the position of the amino group, with or without inorganic salt also present, and of the chain length of the hydrocarbon chain is shown in the following figures. α-Alanine resembles the behaviour of glycine in freezing experiments in that it was not protective if added alone to a thylakoid suspension (Fig. 2). However, β-alanine provided considerable protection, even though it was less effective than sucrose, which has been used as a reference throughout the freezing experiments. Depending on the NaCl concentration, photophosphorylation was protected completely by sucrose (*i.e.* was similar to photophosphorylation before

freezing and occasionally higher than photophosphorylation of unfrozen controls owing to slow aging of the unfrozen samples) at concentrations of 0.1 or 0.2 M. The hydrochloride of α,β-diaminopropionic acid (α,β-alanine) occupied a middle position between α- and β-alanine in that it was protective at low but not at high concentrations.

NaCl considerably altered the protective properties of alanines as shown in

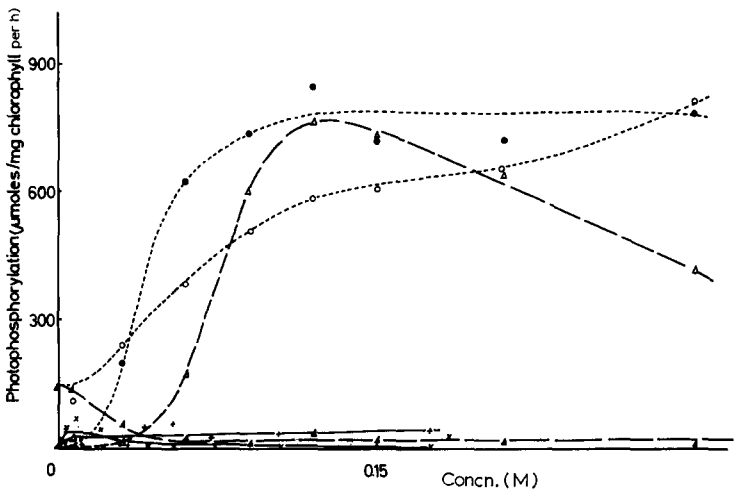

Fig. 1. Protection of washed thylakoids against freezing by glycine, glycylglycine or glycylphenylalanine, with or without NaCl added. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 570 μmoles/mg chlorophyll per h. ▲—▲, glycine; △—△, glycine + 0.1 M NaCl; ○---○, glycylglycine; ●---●, glycylglycine + 0.1 M NaCl; ×—×, glycylphenylalanine; +—+, glycylphenylalanine + 0.02 M NaCl.

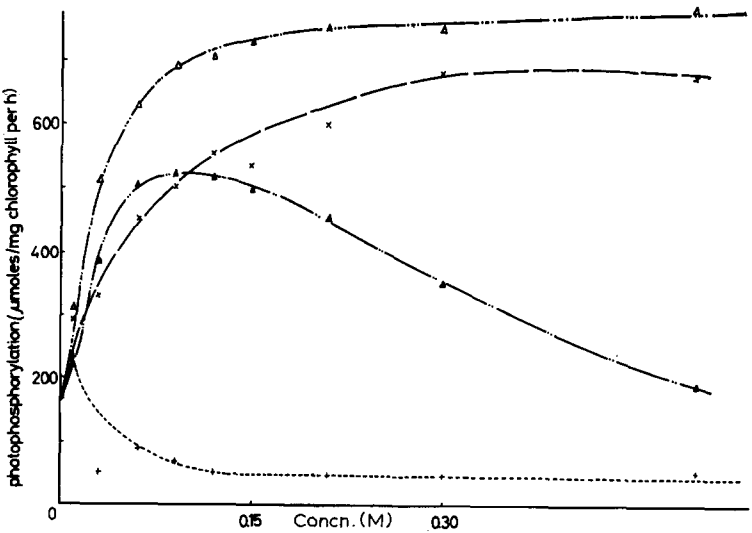

Fig. 2. Protection of washed thylakoid membranes against freezing by α-alanine, β-alanine and the hydrochloride of α,β-diaminopropionic acid (α,β-alanine). Protection by sucrose is shown for comparison. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 430 μmoles/mg chlorophyll per h. +---+, α-alanine; ×—×, β-alanine; ▲-·-·-▲, α,β-alanine; △-·-·-△, sucroce.

Fig. 3. With 0.1 M NaCl present in the system α-alanine protected thylakoids completely at a concentration of 0.15 M, but much less so at lower or higher concentrations. As compared with Fig. 2, the effectiveness of β-alanine was decreased at low concentrations, while considerable protection was still provided at higher concentrations. α,β-Alanine was scarcely protective in the presence of NaCl. It is significant that NaCl-suppressed protection by sucrose was greater than its action against the protection by equimolar amounts of β-alanine (compare Figs 2 and 3).

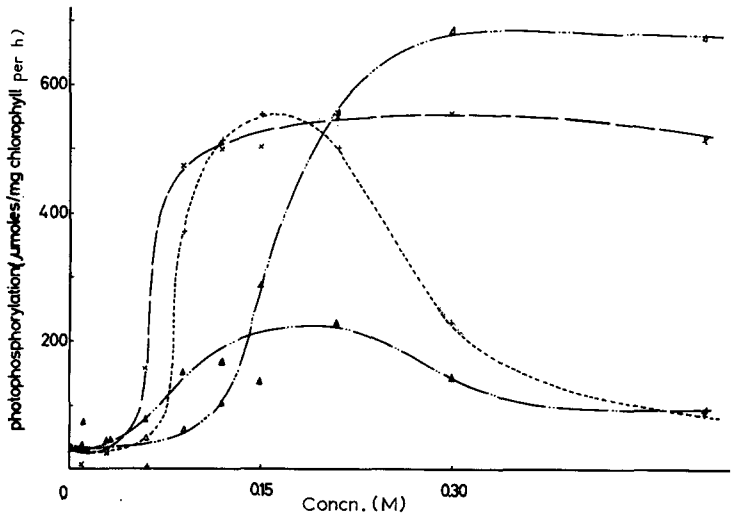

Fig. 3. Effect of NaCl on protection against freezing of washed thylakoid membranes by α-alanine, β-alanine, α,β-alanine (hydrochloride) and sucrose. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 430 μmoles/mg chlorophyll per h. +----+, α-alanine + 0.1 M NaCl; ×--×, β-alanine + 0.1 M NaCl; ▲-·-·-▲, α,β-alanine + 0.1 M NaCl; △-·-·-△, sucrose + 0.1 M NaCl.

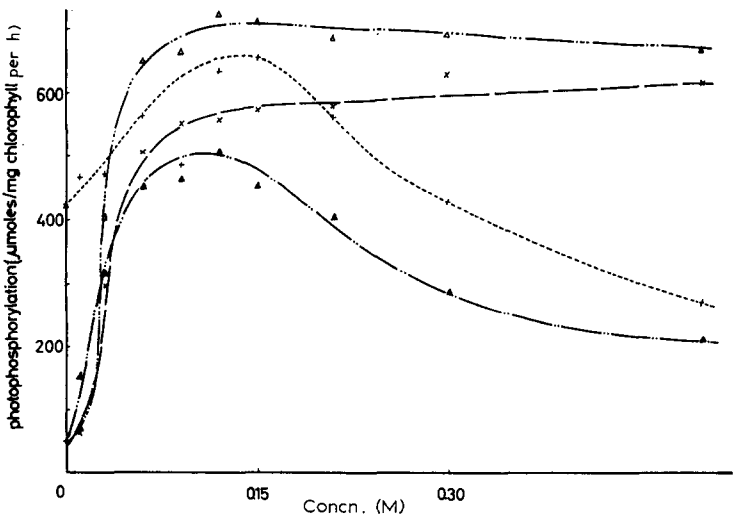

Fig. 4. Protection of washed thylakoid membranes by combinations of α- and β-alanine, α,β-alanine and sucrose. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 430 μmoles/mg chlorophyll per h. +---+, α-alanine + 0.1 M β-alanine; ×--×, β-alanine + 0.1 M α-alanine; ▲-·-·-▲, α,β-alanine + 0.1 M α-alanine; △-·-·-△, sucrose + 0.1 M α-alanine.

The stimulating effect of NaCl on the protective action of α-alanine obviously is not specific, since a combination of β-alanine in a concentration insufficient for complete protection with varying concentrations of α-alanine resulted in almost full protection of the thylakoids at low α-alanine levels (Fig. 4). In the presence

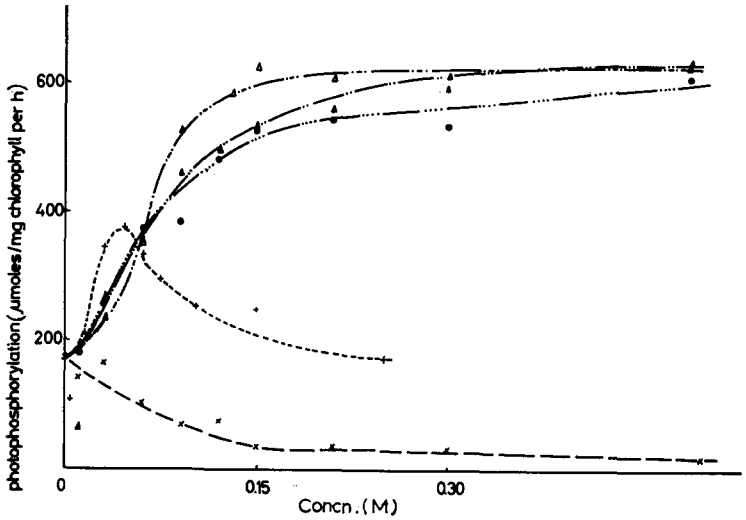

Fig. 5. Protection of washed thylakoids against freezing by α-, β- and γ-aminobutyric acid and by α,γ-diaminobutyric acid (monohydrochloride) as compared with that by sucrose. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 400 μmoles/mg chlorophyll per h. ×—×, α-aminobutyric acid; ●-····-●, β-aminobutyric acid; ▲-··-▲, γ-aminobutyric acid; +---+, α,γ-diaminobutyric acid; △-·-·-△, sucrose.

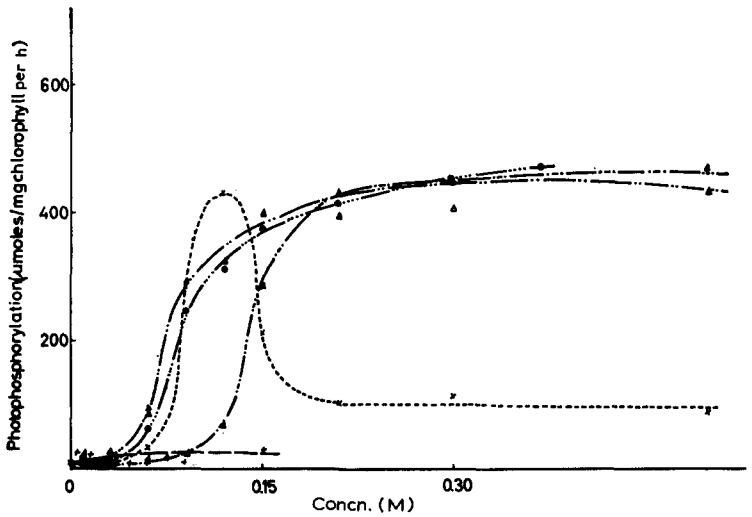

Fig. 6. Protection of washed thylakoids against freezing by α-, β- and γ-aminobutyric acid, and by α,γ-diaminobutyric acid (monohydrochloride) in the presence of 0.1 M NaCl as a function of aminobutyric acids concentration. Protection by sucrose is shown for comparison. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 400 μmoles/mg chlorophyll per h. ×---×, α-aminobutyric acid + 0.1 M NaCl; ●-·-●, β-aminobutyric acid + 0.1 m NaCl; ▲-·-·-▲, γ-aminobutyric acid + 0.1 M NaCl; +—+, α,γ-diaminobutyric acid + 0.1 M NaCl; △-·-·-△, sucrose + 0.1 M NaCl.

of 0.1 M α-alanine protection of photophosphorylation by β-alanine or sucrose was decreased at low, but not at higher concentrations of the latter compounds (Fig. 4).

In principle amino butyric acids behave similarly to alanines. α-Aminobutyric acid increased the freezing damage in the absence of (Fig. 5), but was protective within a certain concentration range in the presence of NaCl (Fig. 6). β- and γ-aminobutyric acids were as protective as sucrose without and even more protective in the presence of NaCl (compare Figs 5 and 6). Depending on the NaCl concentration, α,γ-diaminobutyric acid (hydrochloride) was scarcely, if at all, effective.

A further increase in the chain length completely abolished effectiveness of hydrocarbonic acids having an amino group in the α-position. α-Aminocaproic acid, like valine, leucine and isoleucine, was not protective even in the presence of NaCl. With the amino group further removed from the carboxyl group, effectiveness returned. ε-Aminocaproic acid was as protective as sucrose in the absence of NaCl. In its presence protection was reduced, but less so than with sucrose as protective agent (Fig. 7).

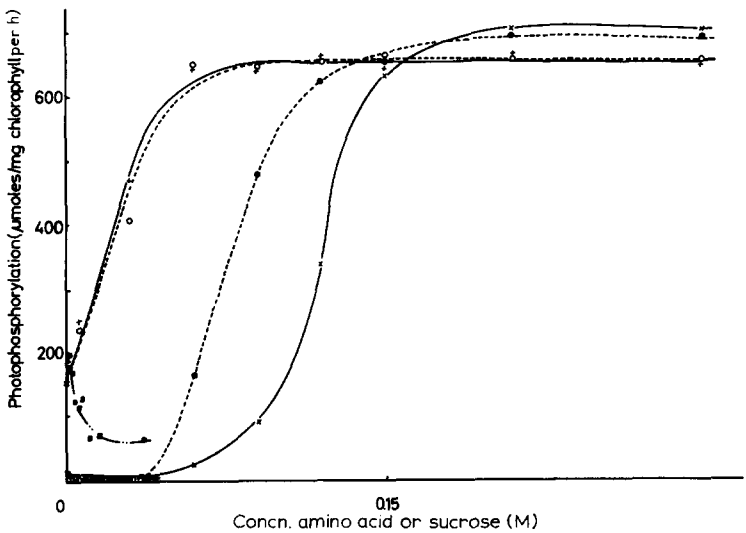

Fig. 7. Effect of α-aminocaproic acid (norleucine) and of ε-aminocaproic acid, with and without NaCl added during freezing of washed thylakoid membranes. Protection by sucrose is shown for comparison. Freezing time 4 h at −25 °C. Photophosphorylation at 0 °C about 680 μmoles/mg chlorophyll per h. ■–·–■, α-aminocaproic acid; □–·–□, α-aminocaproic acid + 0.1 M NaCl; ○---○, ε-aminocaproic acid; ●---●, ε-aminocaprolic acid + 0.1 M NaCl; +—+, sucrose; ×—×, sucrose + 0.1 M NaCl.

DISCUSSION

Protection of thylakoids against the effects of freezing as observed in this work can most easily be explained by unspecific colligative action of cryoprotectants combined with specific effects. This will become clear from a brief consideration of the data shown in Fig. 3. NaCl is known to damage thylakoid membranes at elevated concentrations, whether or not the system is frozen[9,10]. Freezing of thylakoids suspended in 0.1 M NaCl mainly serves to concentrate the system as water is removed by ice formation. The increased salt concentration in the unfrozen part of the system

leads to inactivation of the membranes. The extent of dehydratation, or concentration will, above the eutectic temperature, be a function of the freezing temperature. If a nontoxic solute is present besides NaCl, it will also be concentrated during freezing. Since the freezing temperature determines the total concentration of solute in the unfrozen membrane suspension, which is in or close to equilibrium with ice, the actual salt concentration will be a function of the ratio of nontoxic solute to toxic salt. If this ratio is high enough, the salt concentration will remain so low during freezing as not to damage the membranes. In consequence, protection will be observed. Points of 50 % protection in Fig. 3 occurred at a ratio of organic solute to NaCl of approx. 0.75 for α- and β-alanine; of 1.6 for sucrose; and of 2.7 for an excess of α-alanine. 50 % protection was never reached with the hydrochloride of α,β-alanine. If protection were caused solely by colligative, unspecific action of cryoprotectants, sucrose should be as effective as, for instance, β-alanine, which is not the case. This observation, in addition to others, suggests the assumption of specific effects contributing to protection.

There is the question why some of the amino acids effectively protected photophosphorylation against inactivation during freezing in the absence of NaCl and less effectively in its presence, while others were protective only when NaCl was present in a certain ratio to the amino acid and still others under no circumstances prevented damage to the membranes. The most reasonable explanation is that protection is observed whenever the added solute is nontoxic to the membranes and soluble enough not to crystallise during freezing, since crystallisation would remove it from the system and decrease effectiveness. The results show that amino propionic acids, amino butyric acids and amino caproic acids having an amino group in any except the α-position satisfy these requirements. They protect well in the absence of added salt and in its presence whenever the ratio of amino acid to salt is high enough to prevent salt damage to the membranes. α-Amino acids such as glycine, α-alanine, α-aminobutyric acid and α-aminocaproic acid, on the other hand, are ineffective when added alone to washed thylakoid membranes. They may even increase the damage. In the presence of salt only the α-amino acids having a short aliphatic side chain are protective. Figs 1 and 3 show that glycine and α-alanine have in the presence of 0.1 M NaCl a rather broad range of protection, while that of α-amino butyric acid (Fig. 6) is considerably smaller. α-Aminocaproic acid is not protective in any combination with salt (Fig. 7). Obviously these amino acids are either membrane toxic at exessive concentrations reached during freezing in the absence of salt or when the ratio of amino acid to salt is high, or their solubility is such as not to permit any protection during freezing. Of course, both possibilities may be realised in the same instance.

The low effectiveness as cryoprotectants of the hydrochlorides of 2,3-diaminopropionic acid and of 2,4-diaminobutyric acid (Figs 2, 3, 5 and 6) is probably due in part to the α-position of the amino groups and in part to the chloride anion which is known to be membrane toxic at high concentrations[9].

Not only the length and the polarity of the side chain influence protective properties of amino acids. Fig. 1 shows that peptide formation may lead to increased protection. While in the absence of added NaCl glycine even contributes to damage, glycylglycine is protective. With NaCl present, less glycylglycine is necessary for protection as compared with glycine and no inactivation of photophosphorylation is

observed at high ratios of glycylglycine to NaCl. However, even with peptides the nature of the side chain is important. In contrast to glycylglycine glycylphenylalanine is not protective in the freezing test.

It appears that the dielectric increment, a measure of polarity, may be useful as an approximate criterion of whether and to what extent amino acids may be protective during freezing of biological membranes. The dielectric increments of the α-amino acids tested range between 22.6 and 23.2, that of the β-, γ-, ε-amino acids from 32.4 to 77.5[11]. Glycine, with its limited capacity to act as a protective agent, has a dielectric increment of 22.6; glycylglycine, which is much better suited, 70. However, the importance of dielectric increments should not be overstressed. Glycylphenylalanine, having an dielectric increment of 70.4, is not protective in the freezing test, while proline with a dielectric increment of 21 is a good cryoprotectant. It has been shown elsewhere[5,6], that proline and hydroxyproline and threonine and serine characteristically differ in their protective properties even though they are structurally closely related. This may be viewed as another example of the influence of molecular structure on cryoprotection.

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