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Effects of α-tocopherol (vitamin E) on the stability and lipid dynamics of model membranes mimicking the lipid composition of plant chloroplast membranes

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Abstract **Tocopherol (vitamin E) is widely recognized as a cellular antioxidant. It is essential for human and animal health, but only synthesized in photosynthetic organisms, where it is localized in chloroplast membranes. While many studies have investigated non-antioxidative effects of tocopherol on phospholipid membranes, nothing is known about its effects on membranes containing chloroplast glycolipids. Here, liposomes resembling plant chloroplast membranes were used to investigate the effects of α-tocopherol on vesicle stability during freezing and on lipid dynamics. α-Tocopherol had a pronounced influence on membrane dynamics and showed strong interactions in its effects on membrane stability during freezing with the cryoprotectant sucrose. α-Tocopherol showed maximal effects at low concentrations (around 2 mol%), close to its contents in chloroplast membranes.**

Keywords: α-Tocopherol; Chloroplast lipid; Compatible solute; Freezing stress; Liposome; Vitamin E

1. Introduction

Vitamin E (tocopherols and tocotrienols) is an essential vitamin for humans and animals. However, it is exclusively synthesized in photosynthetic organisms [1]. It is a lipid-soluble antioxidant and in green plant tissues it is localized in the chloroplast envelope and thylakoid membranes, but also in the plastoglobuli, small structures within the chloroplasts, attached to the thylakoid membranes and composed of lipids and proteins [2,3].

α-Toc is a major component of vitamin E in the leaves of higher plants. It is an efficient antioxidant and through several genetic studies it has been shown to play an essential role in protecting the photosynthetic apparatus of plants against oxidative damage under stress conditions [4]. However, studies in various non-plant organisms have shown that α-Toc also has other, non-antioxidative functions [5].

Since α-Toc is a membrane lipid, direct effects on the physical properties of membranes are an obvious possibility. Studies using pure lipid systems have shown that α-Toc has dramatic effects on lipid membrane properties, such as phase behavior and lipid dynamics. In general, these studies have shown that the presence of α-Toc in phospholipid bilayers decreases the motional freedom of the lipid fatty acyl chains in the liquid-crystalline state, which is the predominant state of lipids in biological membranes under physiological conditions. In membranes made from non-bilayer lipids such as phosphatidylethanolamine, on the other hand, non-bilayer structures such as hexagonal II phases are promoted by the presence of α-Toc (see [6,7] for reviews).

Interestingly, while in plants α-Toc is localized in membranes containing only a small fraction of phospholipids, all biophysical studies published to date were performed on pure phospholipid membranes. However, to understand the role of α-Toc in its native membranes these studies are only of limited relevance. Chloroplast membranes only contain a small fraction of phosphatidylglycerol and are mainly (to around 90%) composed of the plant specific glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) [8]. These diacyl lipids contain no phosphate in their headgroups, but instead one (MGDG) or two (DGDG) galactose residues. SQDG has a sulfated glucose moiety as a headgroup and is therefore negatively charged. While DGDG and SQDG are bilayer-forming lipids, pure MGDG forms hexagonal II or cubic phases, depending on the experimental conditions [8,9].

Pure phospholipid membranes may therefore not be a relevant model system to study the effects of α-Toc on the physical behavior of its native plant membranes. In addition, most of the published biophysical studies have used unphysiologically high α-Toc concentrations (20–50 mol%), while the α-Toc content in thylakoid membranes has been shown to be around 2 mol% [3].

Here, we have used liposomes made from a mixture of lipids similar to that found in chloroplast thylakoid membranes and containing 0–10 mol% α-Toc. We have investigated the effect of α-Toc on membrane stability during freezing and on the physical properties of the membrane surface and the

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Abbreviations: CF, carboxyfluorescein; DGDG, digalactosyldiacylglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; EPG, egg phosphatidylglycerol; MC540, merocyanine 540; MGDG, monogalactosyldiacylglycerol; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine; Rh-PE, N-(lissamine Rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine; SQDG, sulfoquinovosyldiacylglycerol; TEN, TES–EDTA–NaCl buffer; TMA-DPH, trimethylammonium-DPH

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hydrophobic interior as a function of the concentration of the cryoprotective sugar sucrose. The results revealed complex interactions between α-Toc and sucrose, which strongly influence membrane stability and lipid dynamics.

2. Materials and methods

2.1. Materials

Egg phosphatidylglycerol (EPG) and α-Toc were purchased from Sigma. N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (NBD-PE), N-(lissamine Rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine (Rh-PE), 1,6-diphenyl-1,3,5-hexatriene (DPH), trimethylammonium-DPH (TMA-DPH), merocyanine 540 (MC540), and carboxyfluorescein (CF) were obtained from Invitrogen (Karlsruhe, Germany). CF was purified according to [10]. The chloroplast glycolipids SQDG, MGDG, and DGDG were purchased from Lipid Products (Redhill, Surrey, UK).

2.2. Preparation of liposomes for leakage and fusion measurements

Lipids were mixed in chloroform, dried under a stream of N_2 and stored under vacuum for at least 2 h to remove traces of solvent. Liposomes for leakage studies were made as previously described [11]. Briefly, an appropriate amount of lipid was 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 ([12]; Avestin, Ottawa, Canada) with 100 nm pore filters. To remove external CF, the liposomes were passed through a Sephadex G-25 column (NAP-5, Pharmacia) in 10 mM TES, 0.1 mM EDTA and 50 mM NaCl (TES–EDTA–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 as described previously [11,13].

Equal volumes of liposomes (10 mg lipid/ml) and solutions containing the appropriate concentrations of sucrose in TEN were combined (40 μl/sample) to reach the final sucrose concentrations indicated in the figures. Samples were rapidly frozen in an ethylene glycol bath precooled to −20 °C [14]. After 2 h of incubation, samples were warmed quickly to room temperature in a water bath.

2.3. 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 by measuring fluorescence at room temperature with a Kontron SFM 25 fluorometer (Kontron Instruments, Neufahrn, Germany) at excitation and emission wavelengths of 460 nm and 550 nm, respectively [11].

Liposome fusion was determined as described before [11,13]. 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 concentrated sucrose solutions in the same manner as for the leakage experiments. Fusion was measured by fluorescence resonance energy transfer [15] with a Kontron SFM 25 fluorometer at excitation and emission wavelengths of 450 and 530 nm, respectively.

2.4. Partitioning of merocyanine 540 into liposome membranes

To assess the effects of α-Toc on the surface properties of membranes, we used the dye MC540 as described previously [16,17]. Liposomes (0.3 mg/ml) were suspended in TEN containing the appropriate concentrations of sucrose. Samples were incubated at 0 °C for 30 min and then MC540 was added to a final concentration of 10 μM. After 15 min, the absorbance was measured at 570 nm and 530 nm in an Uvikon 922 double beam spectrophotometer (Kontron Instruments) at room temperature. The reference cuvette contained liposomes and sucrose without MC540.

2.5. Steady-state anisotropy of membrane lipids

The dynamics of lipids as a function α-Toc and sucrose concentrations were determined by measuring the degree of depolarization of the fluorescence emitted from the probes DPH, TMA-DPH, and NBD-PE [17,18]. DPH is a hydrophobic molecule and is widely used for measuring the order of the lipid fatty acyl chains in the core region of the bilayer, while TMA-DPH is anchored at the water–lipid interface, due to the additional charged trimethylammonium group [19,20]. NBD-PE reports on the mobility of the lipid headgroup region of the membranes [21]. DPH or TMA-DPH in dimethyl formamide was added to a liposome suspension (0.1 mg/ml) in TEN containing up to 1 M sucrose in a cuvette at room temperature (approximately 22 °C). The lipid/probe molar ratio was 200/1 and the final dimethyl formamide concentration was 0.1% (v/v). Measurements were carried out in a PerkinElmer LS55 spectrofluorimeter with polarization filters. Fluorescence was excited at 360 nm and emission was recorded at 450 nm. NBD-PE in chloroform was mixed with the other lipids at a lipid/probe molar ratio of 200/1 and liposomes were prepared in TEN by extrusion as described above. Fluorescence depolarization was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. From the polarization values, the dimensionless anisotropy was calculated as described recently [22].

3. Results and discussion

In all experiments, liposomes were composed of 40 mol% MGDG, 30 mol% DGDG, 15 mol% SQDG, 15 mol% EPG and α-Toc up to 10 mol%. The proportion of the different diacyl lipids between each other was held constant, so that the addition of α-Toc only changed the ratio between total diacyl lipids and α-Toc. This led to a lower fraction of diacyl lipids with increasing α-Toc content. For the sake of clarity and brevity only the diacyl lipid composition in the absence of α-Toc is shown, along with the appropriate α-Toc content.

In the first set of experiments, liposomes were frozen to −20 °C in the presence of different concentrations of sucrose, a well-characterized cryoprotectant for both native thylakoid membranes [23] and liposomes composed of thylakoid membrane lipids [22]. After thawing, damage to the membranes was assessed either as leakage of the soluble fluorescent dye CF from the vesicle interior, or as liposome membrane fusion (Fig. 1). As in a previous study [22], a wide range of sucrose concentrations (0–1 M) was used in these experiments, although such high concentrations are not found in vivo. However, on the one hand, these experiments were designed to elucidate particular biophysical effects and therefore, sufficiently high concentrations should be used to obtain clear effects and to elucidate possible concentration dependencies. On the other hand, it has been shown that the freeze-thaw stability of thylakoid membranes is influenced by several different compounds that are present in the chloroplast stroma, such as divalent cations and various amino acids [24], so that sucrose can only function as an example of a possible and well-characterized cryoprotectant.

In the absence of sucrose, α-Toc had no effect on CF leakage, while membrane fusion was reduced by about 20% at the highest α-Toc concentration in the membranes (10 mol%). In contrast, the cryoprotective effects of sucrose were strongly modulated by α-Toc. Sucrose only had a moderate protective effect on both CF leakage and membrane fusion in chloroplast lipid membranes without α-Toc, but the inclusion of only 1 mol% α-Toc induced a dramatic reduction of fusion already at moderate sucrose concentrations. Increases in α-Toc content up to 10 mol% further increased the protective effect of sucrose on membrane fusion during freezing.

The effect of α-Toc content on the protection of liposomes from CF leakage during freezing by sucrose was more complex. While low amounts of α-Toc (0.5–2 mol%) increased