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Changes in physical properties of vacuolar membrane during transformation of protein bodies into vacuoles in germinating pumpkin seeds

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Abstract

Changes in membrane molecular dynamics associated with the transformation of protein body membranes into vacuolar membranes during pumpkin seed germination, were monitored by EPR-spin probe technique. Using highly purified membrane preparations as well as 5-SASL and 16-SASL spin labels, parameters like general membrane lipid fluidity, order parameter, semicone angle, rotational correlation times τ_{2B} and τ_{2C}, ratio of immobilized to mobile lipids were determined and the activation energy for rotational diffusion of 16-SASL was calculated. Analysis of these parameters at different temperatures indicated a more rigid nature of protein body membrane comparing to vacuolar membrane, as a result of a more restricted motional freedom of lipids. These differences are discussed in terms of protein composition and various functional specialization of both types of membranes.

Keywords: EPR; Membrane; Molecular dynamics; Protein body; Spin label; Transformation; Vacuole

1. Introduction

Various seed reserve proteins are stored in the protein bodies that are widely distributed in the seed cells of higher plants. Seed proteins are actively synthesized as precursor forms on rough endoplasmic reticulum and then transported via dense vesicles to vacuoles during seed maturation [1–4]. The precursors of seed proteins are converted into their mature forms by a specific vacuolar processing enzyme and accumulated in the vacuoles [2,5–8].

The vacuoles appearing in maturing seeds are regarded as protein storage vacuoles and are distinguished from vegetative vacuoles that are designated as lytic compartments, containing a variety of hydrolases [9]. Protein bodies are formed from the vacuoles at the late stage of seed maturation and are surrounded by a membrane deriving from vacuolar membrane [10].

When germination starts after seed imbibition, a reverse process is observed. Protein bodies undergo a fusion, creating larger vesicles that finally form a single, central vacuole of the cell [11]. Since protein body membranes and vacuolar membranes differ in their apparent functional specialization, it is plausible to assume that this difference should be reflected by changes in composition and properties of both types of membranes.

This morphological transformation is accompanied by the breakdown of the protein constituents of protein bodies and the incorporation of newly synthesized proteins into the vacuoles. The degradation of protein body components includes the breakdown of the membrane proteins [12–14]. Five major membrane proteins, designated as MP23, MP27, MP28, MP32 and MP73, were detected in the protein body membrane of pumpkin [13]. Both MP23 and MP28 are seed specific α-TIP (tonoplast intrinsic protein) that are widely conserved among dicots [13,15,16]. Although all the five membrane proteins finally disappear during transformation of protein bodies into vacuoles, the peripheral

Abbreviations: 5-SASL, 5-doxylstearic acid; 16-SASL, 16-doxylstearic acid; EPR, electron paramagnetic resonance.

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membrane proteins MP27 and MP32, originating from a single precursor, disappear most rapidly [13]. Recently Maeshima et al. [14] reported that vacuolar H^+-pyrophosphatase, H^+-ATPase and γ-TIP are newly synthesized and are incorporated into the vacuolar membrane during organellar transformation.

The transformation of protein body membranes into vacuolar membrane is accompanied by distinct changes in the composition of both peripheral and integral membrane proteins as described above. These changes may reflect different functions and processes occurring in both types of membrane. Since the membrane protein composition is modified during protein body-vacuole transformation, one may expect some changes in certain physical parameters of the membrane undergoing such transformation. Occurrence and effectiveness of various membrane-related processes and functions strictly depend on membrane physical properties like membrane fluidity, order parameter of membrane lipid fatty acids etc. [17–25], therefore we undertook studies on changes in membrane molecular dynamics accompanying protein body membrane-vacuolar membrane transformation.

2. Materials and methods

2.1. Plant material

Pumpkin (*Cucurbita* sp. Kurokawa Amakuri Nankin) seeds were soaked overnight in tap water and then germinated and grown on Vermiculite at 25°C for 4 days in darkness.

2.2. Isolation of protein body membranes

Pumpkin dry seeds shell free were blended with a knife homogenizer (Universal Homogenizer, Nihon Seiki Seisakusho, Tokyo, Japan) 3×5 s at full speed. Homogenization was continued with mortar and pestle with successive addition of anhydrous glycerol. After filtration through a double layer of cheese cloth the homogenate was centrifuged in a Beckman JA-20 rotor at 12000 rpm for 15 min (at 15°C). The pellet containing protein bodies was resuspended in glycerol and centrifugation was repeated as above. To disrupt protein bodies and liberate protein crystalloids the pellet was suspended in 10 mM Tris-Mes buffer, pH 6.5 (osmotic shock) and subjected to sonication (4×15 s, output set to 4, sonicator Branson Sonifier Cell Disruptor 200, Branson Sonic Power, Danbury, Conn, USA). From this step on all operations were carried out at 4°C.

Liberated protein body membranes were separated from storage proteins by centrifugation at 1500 rpm for 15 min and collected in the supernatant. To remove completely the residual crystalloids, the protein body membrane suspension was mixed with 5 M NaCl to give a final concentration of 1 M. Then the suspension was ultracentrifuged on a Beckman ultracentrifuge at 40000 rpm for 1 h using 45Ti rotor. The resulting supernatant was discarded and the pellet of crude protein body membranes was suspended in a small volume of 10 mM Tris-Mes buffer, pH 6.5 and homogenized in a glass-teflon homogenizer.

The crude membrane suspension was further purified by sucrose density gradient (5–60%, w/w) centrifugation (21000 rpm, 2 h) in a Beckman SW28 rotor. A distinct yellow band of protein body membranes was withdrawn, diluted with Tris-Mes buffer and centrifuged at 40000 rpm for 1.5 h using 45Ti rotor. The pellet of purified protein body membranes was resuspended in Tris-Mes buffer.

2.3. Isolation of vacuolar membranes

Cotyledons from 4-day-old, dark-grown pumpkin seedlings were collected and gently chopped on ice with a rasor blade, gradually adding 10 mM Tris-Mes buffer, pH 6.5, containing 0.5 M mannitol. The homogenate was filtered through two layers of cheese-cloth and the remaining solid residue was transferred to a prechilled mortar, homogenized with the same buffer and again filtered through cheese-cloth.

Combined filtrates were layered on the top of 40% Percoll in 0.5 M mannitol and 10 mM Tris-Mes buffer, pH 6.5 and centrifuged at 1800 rpm for 10 min in a horizontal type rotor CD-100R (Tomy Seiko, Tokyo, Japan). The supernatant was discarded and the pellet containing vacuolar membranes was suspended in Tris-Mes buffer, vortexed and sonicated two times for 30 s with a 30 s interval in between. The power output was set on 4. After sonication the suspension was filtered through miracloth and then centrifuged at 2000 rpm for 15 min. The supernatant was diluted with 5 M NaCl to the final concentration of 1 M and centrifuged at 40000 rpm for 1 h using a 45Ti rotor. The pellet was suspended in a small volume of Tris-Mes buffer, homogenized with a glass-teflon homogenizer and layered on top of the sucrose density gradient (10–60%, w/w). After centrifugation at 21000 rpm for 2 h in a SW28 rotor the yellowish band of vacuolar membranes was collected, diluted with Tris-Mes buffer and centrifuged again at 40000 rpm for 1.5 h in a 45Ti rotor. The pellet of purified vacuolar membranes was resuspended in Tris-Mes buffer.

2.4. Spin label EPR measurements

Two spin labels, derivatives of stearic acid were used throughout this study; a 5-doxylstearic acid (5-SASL), reporting on dynamics of membrane regions close to the headgroup area, and 16-doxylstearic acid (16-SASL), giving information on the molecular dynamics of the membrane interior.

Spin labels were incorporated into the membrane preparation by vortexing a membrane sample in a conical tube

with deposited thin film of the respective spin label as described in [17]. Their final concentration in the sample was $(1-1.5)\cdot 10^{-4}$ M. The amount of membranes in a sample corresponded to 300–350 μg of protein. EPR spectra of spin labels as a function of temperature were recorded using a Bruker ER-2000 EPR spectrometer working in X band, using parameters as in Ref. [26]. All measurements on the temperature dependence of membrane molecular dynamics were performed in a heating mode. Both spin labels were purchased from Sigma.

2.5. Protein determination

Protein composition of protein body membranes and vacuolar membranes was determined by SDS-PAGE using a 12.5% gel and the buffer system of Laemmli [27]. A typical polypeptide pattern of both types of membrane was found (data not shown). Protein concentration was measured using Bio-Rad protein assay.

2.6. Light microscopic analysis

For light microscopy, pumpkin cotyledons of dry seeds, 2-day-old seedlings and 4-day-old seedlings were sliced vertically (relative to the axis) and the slices (1–2 mm thick) were fixed in Bouin's solution containing 71% picric acid, 9% formaldehyde and 4.7% acetic acid. Each specimen was embedded in paraffin. Thin sections were cut on a microtome and stained with azocarmine G and aniline blue according to the azan staining procedure [28]. The sections were examined with a light microscope (model BHB; Olympus, Japan).

2.7. Electron microscopic analysis

Electron microscopy of the membrane fractions of protein bodies or vacuoles, purified by sucrose density gradient centrifugation, was carried out essentially by the method of Mettler and Beevers [29]. Each membrane fraction was dispersed in a 1% solution of agar that contained 10 mM Tris-Mes (pH 6.5) and then fixed in 0.25 M sucrose, 2% glutaraldehyde and 0.1 M potassium phosphate (pH 7.2). The samples were washed with 0.1 M potassium phosphate (pH 7.2) and post-fixed with 0.1% OsO_4 in 0.1 M potassium phosphate (pH 7.2) for 16 h. The specimens were dehydrated with a graded acetone series and embedded in Spurr's resin. Ultrathin sections were post-stained with 4% uranyl acetate and 0.4% lead citrate and then observed under a transmission electron microscope (model 1200EX; JEOL, Japan).

3. Results and discussion

Subsequent stages in transformation of protein bodies into vacuoles in germinating pumpkin cotyledons are illustrated in Fig. 1 and the electron microscope photographs of the investigated samples of protein body membranes and vacuolar membranes are shown in Fig. 2.

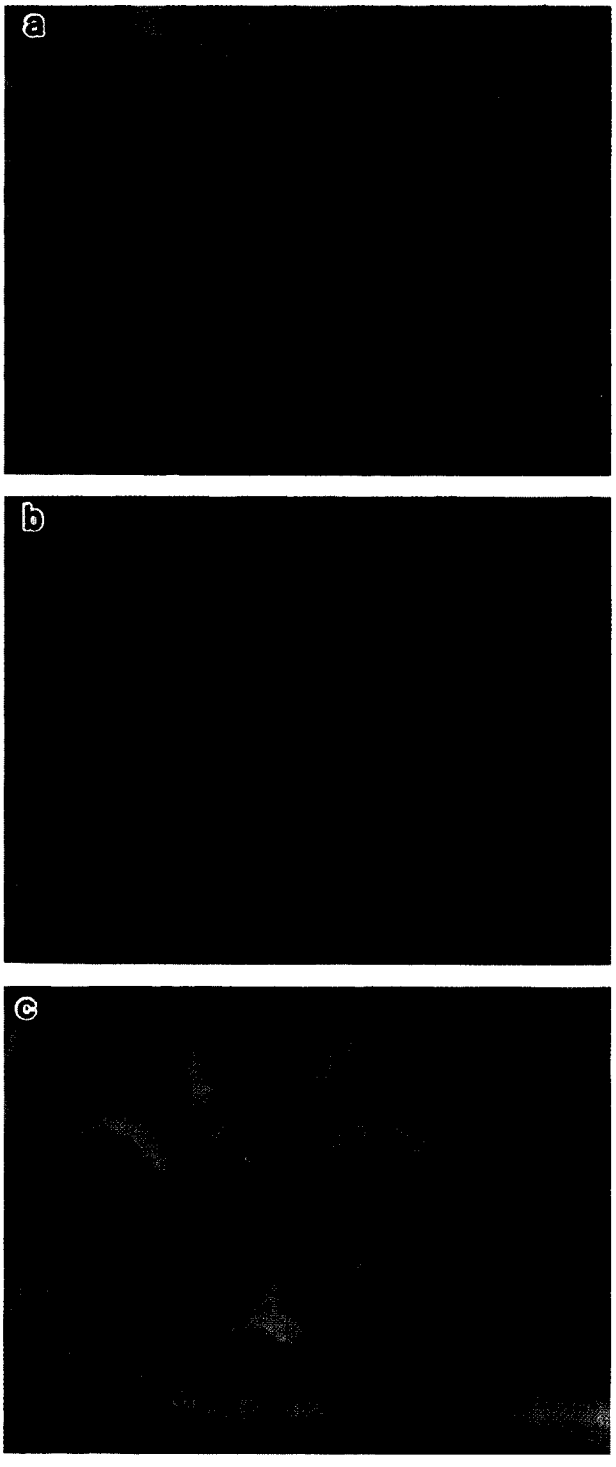

Fig. 1. Light micrographs of pumpkin cotyledonary tissues at different stages of seedling growth: dry seeds (a), 2-day-old seedlings (b) and 4-day-old seedlings (c). Bars = 20 μm.

In this study the application of two types of spin labels, derivatives of stearic acid, having attached the 'reporting' nitroxide group to the 5th (5-SASL) and the 16th (16-

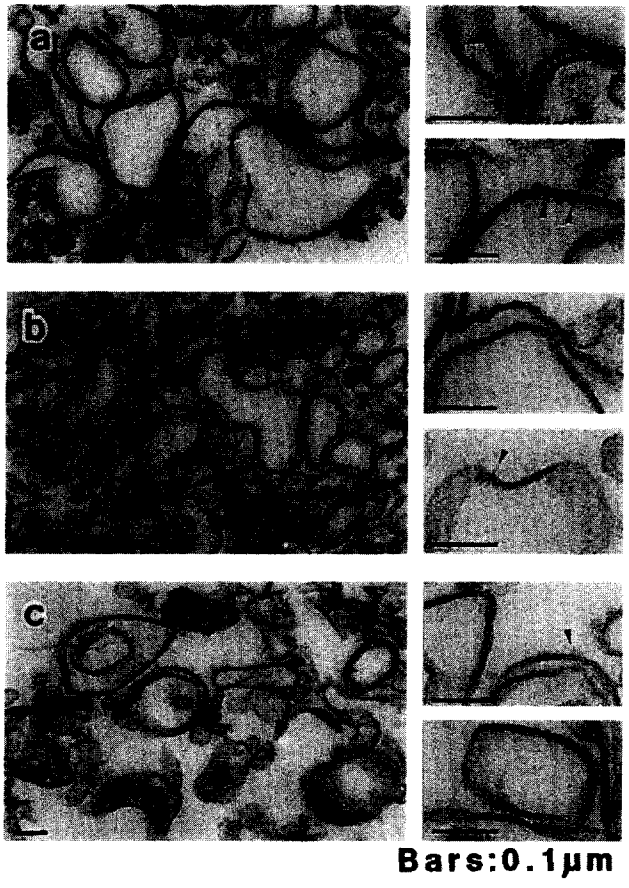

Fig. 2. Electron micrographs of the purified membranes of protein bodies and vacuoles. The densities of the membranes were 1.20 g cm^{-3} for dry seeds (a), 1.17 g cm^{-3} for 2-day-old seedlings (b) and 1.07 g cm^{-3} for 4-day-old seedlings (c). Arrowheads indicate proteins associated with the membranes.

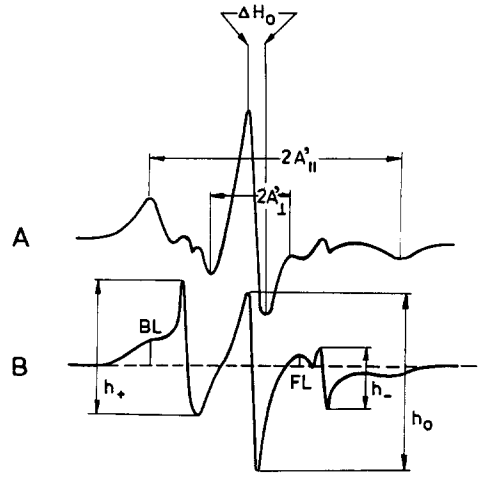

Fig. 3. Schematic representation of EPR spectra of 5-SASL (A) and 16-SASL (B) spin labels with the analyzed spectral parameters indicated. BL and FL-peaks corresponding to spin label molecules localized in strongly immobilized and fluid membrane lipid domains, respectively.

SASL) carbon, respectively, enabled to gain information about molecular dynamics at different depth of the investigated membranes. 5-SASL spin label with its nitroxide group localized close to the membrane lipid head group area reports on the molecular dynamics processes occurring near the membrane surface, whereas 16-SASL with its nitroxide situated close to the membrane midplane, gives an information about dynamic processes occurring in the membrane interior. Fig. 4 shows the temperature dependence of the spectral parameter $2A'_{\|}$ (an outermost splitting, for the explanation see Fig. 3) of 5-SASL as well as of 16-SASL spin labels incorporated into the investigated membranes. This parameter gives information about the general mobility of the spin label in the membrane. It thus reflects the degree of membrane fluidity. As shown in Fig. 4 protein body membranes and vacuolar membranes do not differ greatly in their fluidity at low temperature (−30°C). However, upon temperature increase, the difference in fluidity increases, the vacuolar membranes being more fluid than protein body membranes. The highest differences were observed at the physiological range of temperatures (5–35°C). The increased fluidity of vacuolar membranes in comparison with protein body membranes concerned both the membrane head group zone (5-SASL) and the hydrophobic membrane interior (16-SASL).

Another parameter of the spin label EPR spectrum, which may be applied to the description of reorientational motion of free radical moiety is ΔH_0 (Fig. 3). This parameter combines the information on wobbling of a spin label and its rotation along the long axis of the molecule. As show the data presented on Fig. 5, again vacuolar membranes exhibit greater freedom of spin label motion than protein body membranes at the corresponding temperatures.

For certain ranges of temperatures studied, it is possible

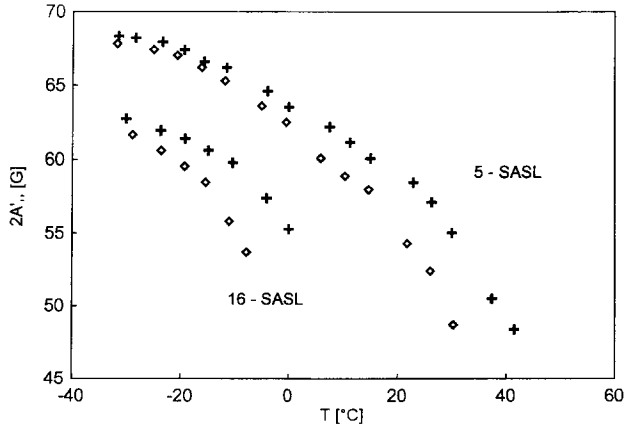

Fig. 4. Temperature dependence of an outermost splitting of 5-SASL and 16-SASL doped into protein body membranes (+) and vacuolar membranes (◇).

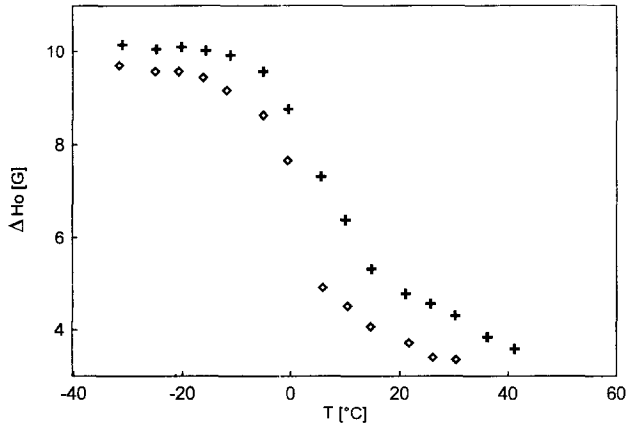

Fig. 5. Temperature dependence of ΔH_0 parameter of 5-SASL doped into protein body membranes (+) and vacuolar membranes (◇).

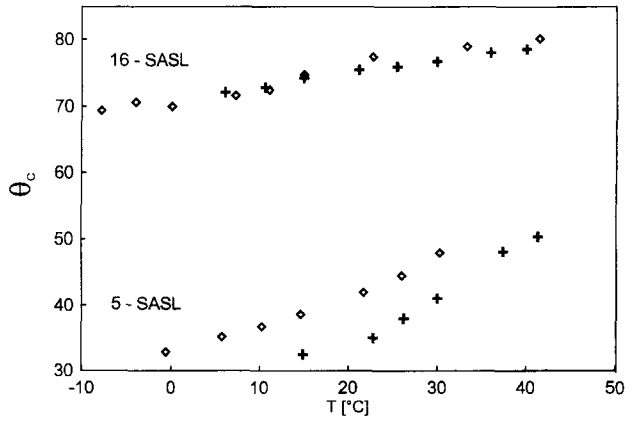

Fig. 7. Temperature effect on semicone angle θ_c of 5-SASL and 16-SASL incorporated into protein body membranes (+) and vacuolar membranes (◇).

to calculate an order parameter 'S' [30] using the following formula [31]

$$S = 0.5407(A'_{\parallel} - A'_{\perp})/a_0 \quad (1)$$

where

$$a_0 = (A'_{\parallel} + 2A'_{\perp})/3 \quad (2)$$

The order parameter, which is related to outermost splitting, may change from the value of 1 (in the case of maximally ordered membranes) to 0 (in the case of the maximum disorder). The data in Fig. 6 show that the environment of spin label is more ordered in the case of protein body membranes, as compared with vacuolar membranes; both near membrane lipid headgroup area (Fig. 6A) as well as in the membrane hydrophobic interior (Fig. 6B).

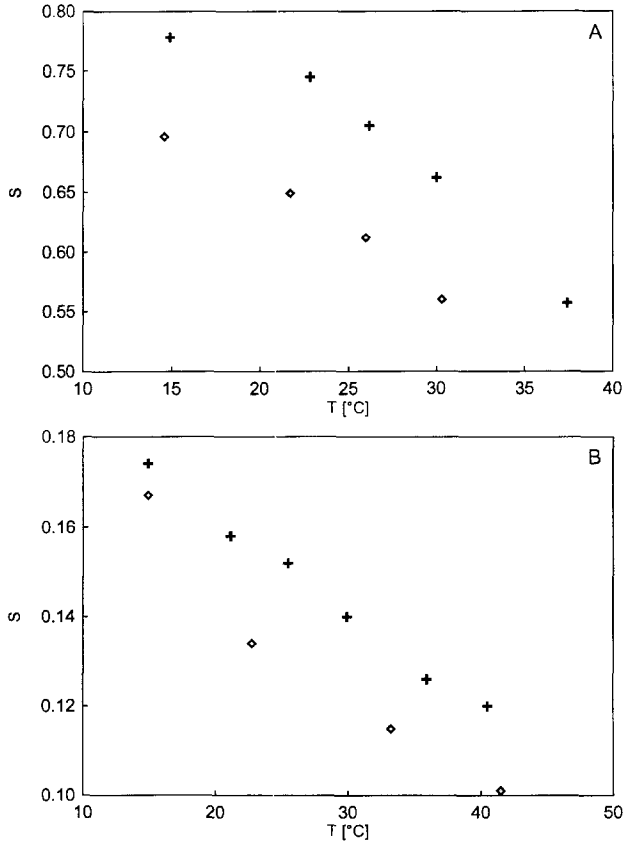

Fig. 6. Changes in order parameter S as a function of temperature, for 5-SASL (A) and 16-SASL (B) incorporated into protein body membranes (+) and vacuolar membranes (◇).

Wobbling of the long axis of the applied spin label molecules occur within a cone which shape is imposed by the surrounding molecules of the membrane constituents. The respective semicone angle θ_c is related to the order parameter S and can be calculated using the following equation [32]

$$S = \cos\theta_c(1 + \cos\theta_c)/2 \quad (3)$$

Semicone angles of 5-SASL and 16-SASL as well as their changes as a function of temperature are shown in Fig. 7. As can be concluded on the basis of these data, 5-SASL in vacuolar membranes exhibits larger semicone angle than in protein body membranes at the same temperatures. This indicates that the spin label has more motional freedom for wobbling type of motion in vacuolar membranes, most probably because of their less dense packing in the region close to headgroup area. There is, however, not much difference in the semicone angle of 16-SASL in both membrane types which points to a high fluidity of membrane midplane area in the temperature range studied.

Application of the fast moving 16-SASL spin label for the EPR measurements permitted calculation of the effective rotational correlation times τ_{2B} and τ_{2C} [33], assuming isotropic rotational diffusion of the spin probe.

$$\tau_{2B} = 6.51 \cdot 10^{-10}\Delta H_0\left((h_0/h_-)^{1/2} - (h_0/h_+)^{1/2}\right)s \quad (4)$$

$$\tau_{2C} = 6.51 \cdot 10^{-10}\Delta H_0\left((h_0/h_-)^{1/2} + (h_0/h_+)^{1/2} - 2\right)s \quad (5)$$

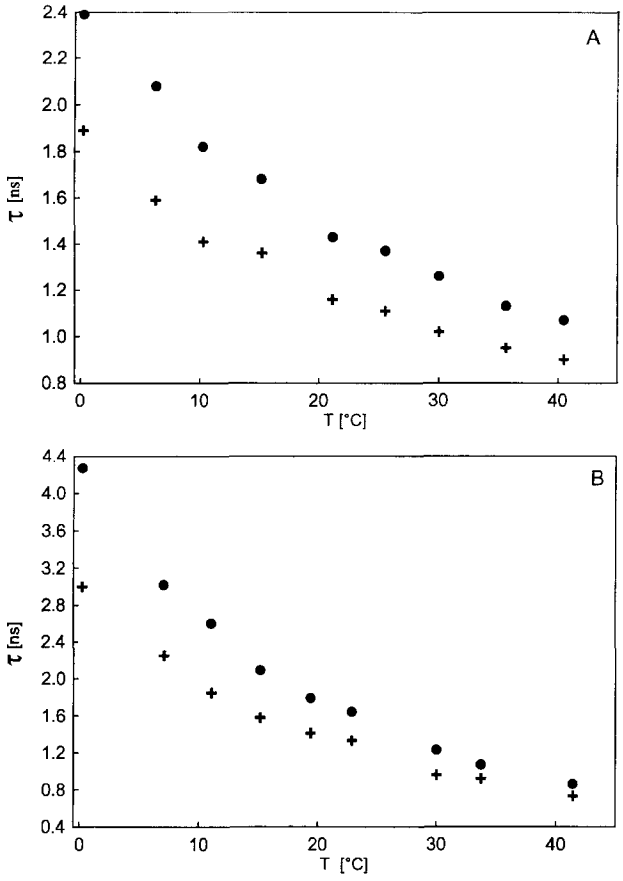

Fig. 8. Temperature dependence of the rotational correlation times τ_{2B} (+) and τ_{2C} (●) for 16-SASL doped into protein body membranes (A) and vacuolar membranes (B).

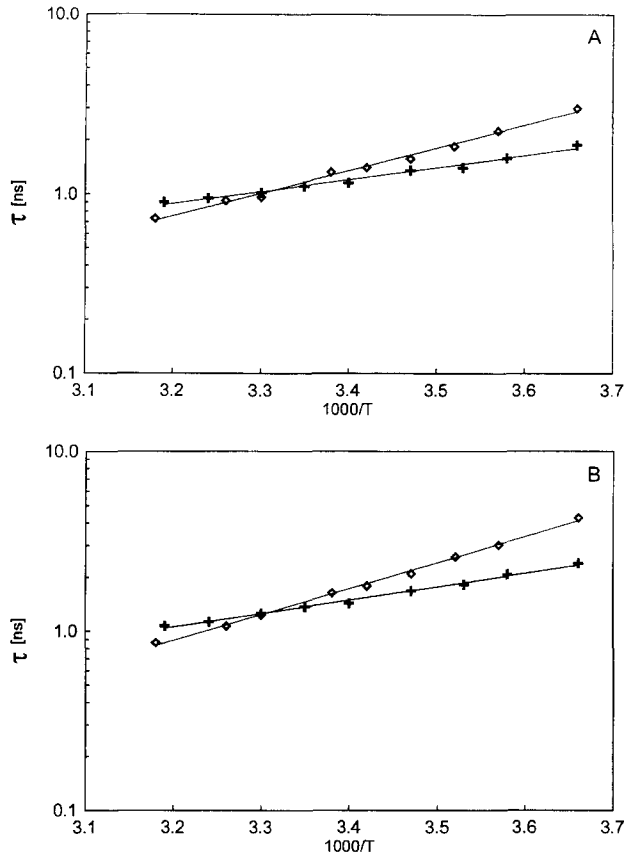

Fig. 9. Arrhenius plot of the correlation times τ_{2B} (A) and τ_{2C} (B) for 16-SASL incorporated into protein body membranes (+) and vacuolar membranes (◇).

where ΔH_0 is the peak-to-peak width of the central line in the spin label spectrum (in gauss) and h_+, h_0 and h_- are the heights of the low, central and high field component of the spectrum, respectively (see Fig. 3). It is assumed that τ_{2B} corresponds to the rotation of the spin label molecule along its long axis while τ_{2C} gives the information about the movement of the molecule in the direction perpendicular to the long axis. Similar values of τ_{2B} and τ_{2C} indicate an isotropic motion of the spin label molecule, while anisotropy of movement reflects in a difference between these correlation times.

The values of τ_{2B} and τ_{2C} of 16-SASL in both kinds of investigated membranes depend on the temperature (Fig. 8A and B). In a low temperature range (around 0°C) the differences between τ_{2B} and τ_{2C} indicate an anisotropic rotational diffusion. A rise in temperature brings about a shortening of both correlation times as well as a decrease of the difference between them, which gives the evidence that the motion becomes more and more isotropic. From Fig. 8 as well as from Fig. 9, which shows an Arrhenius plots of the measured correlation time data (log τ versus $1/T$), it is evident, that for vacuolar membranes below 30°C both τ_{2B} and τ_{2C} are longer than in the case of protein body membranes. However, they become shorter above this temperature, indicating a difference in the activation energy of rotational motion of the nitroxide group of the spin label in both types of membranes. The calculated activation energy for rotational diffusion of 16-SASL both from τ_{2B} and τ_{2C} (Table 1) clearly demonstrates this difference.

At certain temperatures, in the spectra of both 5-SASL as well as 16-SASL, components corresponding to spin label molecules localized in the environment of freely moving lipids and bound (immobilized) lipids can be distinguished (Fig. 3). Using these parameters a ratio of bound lipids to free lipids in the membrane can be estimated. Fig. 10 shows that protein body membranes contain a higher proportion of bound lipids than vacuolar membranes at the same temperatures.

Table 1
Activation energy for rotational diffusion of 16-SASL in protein body membranes and vacuolar membranes

	ΔE (kcal/mol)	
	from τ_{2B}	from τ_{2C}
Protein body membranes	3.08	3.43
Vacuolar membranes	5.84	6.67

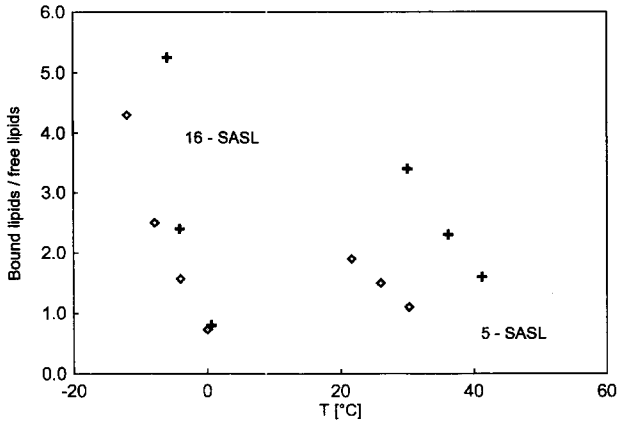

Fig. 10. Ratio of immobilized lipids to freely moving lipids in protein body membranes (+) and vacuolar membranes (◇) at selected temperatures, as measured using 5-SASL and 16-SASL.

All the data presented above indicate pronounced differences in physical parameters of both investigated membrane types. Protein body membranes are less fluid than vacuolar membranes and their lipid constituents exhibit, in general, a more restricted motional freedom. On the other hand, vacuolar membranes seem to constitute rather dynamic system with some physical parameters resembling closely those of thylakoid membranes of chloroplasts (data not shown), which are known for their high degree of fluidity [17,26].

Obviously, the differences in molecular dynamics between protein body membranes and vacuolar membranes reflect their different functions. Protein body membranes seem to play a protective role for the encapsulated storage protein crystalloids. Perhaps such a protective function requires the membrane to be rather rigid. On the other hand the vacuolar membrane is not only the barrier separating the vacuole from the rest of the cell. It is also the site of many active, membrane-localized cellular processes, including several transport systems [34,35]. These systems seem to require rather fluid membrane for their efficient operation, as it is also the case in photosynthetic membranes. It has been recently reported [36] that vacuolar membranes of crassulacean-acid-metabolism plants may also change fluidity in response to such factors as temperature and salinity stress.

The mechanism involved in the changes of molecular dynamics during transformation of protein body membranes into vacuolar membrane is unknown by now and needs a further research. One of the factors contributing to the changes in membrane fluidity during such transformation may be an alteration in the ratio of lipids to membrane proteins, especially integral proteins [26,37,38] which bind surrounding lipids to their exposed hydrophobic amino acid residues. Indeed, the amounts of 23 kDa and 27 kDa integral proteins characteristic for protein body membranes diminish pronouncedly during the transition of protein bodies into vacuole (data not shown). It has been also found recently that transmembrane peptides may cause changes in the lipid domain structure in membranes [39].

Another possible factor controlling molecular dynamics of protein body and vacuolar membranes is the degree of unsaturation of fatty acids of membrane lipids. However, whether indeed this is the case or there is also a contribution from plant sterols or other molecules known to be the modulators of membrane molecular dynamics [40,41] remains to be found. There is only limited published data on lipid composition of vacuolar membranes [42,43] and since (to our knowledge) there is no information available on the lipid composition of protein body membranes, it is not possible to correlate the observed differences in molecular dynamics between the two kinds of investigated membranes with their possible differences in lipid content and degree of fatty acid unsaturation.

There are some data which suggests that sterols may be involved in the changes of membrane physical properties during protein body-vacuole transformation. As demonstrated in [43], tonoplast in etiolated seedlings of mung bean has lower sterol to phosholipid molar ratio than plasma membrane, but higher content of phospholipid on protein basis. This indicates on rather fluid nature of vacuolar membranes. On the other hand, protein body membranes in developing soybean cotyledons exhibit higher sterol content than plasma membrane [44]. The above mentioned data, although obtained for two different plant species indicate that the changes in sterol level may be responsible (at least in part) for the observed changes in membrane molecular dynamics.

Acknowledgements

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