Phase Transitions in Liposomes Formed from the Polar Lipids of Mitochondria from Chilling-Sensitive Plants

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ABSTRACT

The thermal response of mitochondrial polar lipids from a variety of chilling-sensitive and chilling-insensitive plants was determined by differential scanning calorimetry. A phase transition was observed at 15°C for mitochondria from soybean (Glycine max. cv Davis) hypocotyl, at 16°C for tomato (Lycopersicon esculentum cv Flora-Dade and cv Grosse Lisse) fruit, at 15°C for cucumber (Cucumis sativus L.) fruit, at 14°C for mung bean (Vigna radiata var Berken) hypocotyl, and at 15°C for sweet potato (Ipomoea batatas L.) roots. The transition temperature was not significantly altered by the scan rate and was reversible. Changes in the temperature coefficient of motion for a spin label, intercalated with the polar lipids, occurred at a temperature slightly below that of the phase transition, indicating that the polar lipids phase separate below the transition. No phase transition was observed for mitochondrial polar lipids from barley (Hordeum vulgare) roots, wheat (Triticum aestivum L. cv Falcon) roots, and Jerusalem artichoke (Helianthus tuberosus L.) tubers. The results show that a phase change occurs in the membrane lipids of mitochondria a few degrees above the temperature below which chilling injury is evident in the sensitive species. Thus they are consistent with the hypothesis that sensitivity to chilling injury is related to a temperature-induced alteration in the structure of cell membranes.

Many of the enzymes associated with the electron transport system of mitochondrial membranes from chilling-sensitive plants show an abrupt increase in Arrhenius activation energy below about 10°C (10, 16). For mitochondria from chilling-insensitive plants the same enzymes show a constant E in between 0°C and 25°C (10, 16, 18). The temperature for the change in E of the enzyme systems of chilling-sensitive plants correlates with that of a thermally induced, structural change in the membrane lipids of the mitochondria. This structural change has been detected by both ESR and fluorescence spectroscopy using aqueous dispersions of the membrane polar lipids (16, 17, 21). Based on this correlation between structure and function it was proposed that the change in the thermal response of these enzymes was induced by the phase transition in the membrane lipids (10, 16, 21). Furthermore, since the change in structure and function of the membranes occurs at a temperature below

which some plants develop symptoms of chilling injury, it was proposed that the primary event initiating chilling injury is the phase change in the membrane lipids (9, 10).

Calorimetric studies have established that a phase change occurs in the polar lipids of chloroplast membranes of several species of chilling-sensitive plants at temperatures which correlate with the onset of chilling injury (22, 24). However, there is some controversy regarding whether phase transitions occur in the membranes of mitochondria from chilling-sensitive plants and how these relate to chilling injury. For example, an investigation using calorimetry and light-scattering failed to detect a phase transition above 0°C in the polar lipids of mitochondria from soybean, a chilling-sensitive plant (14). In another study, the authors reported a broad exothermic transition, starting at about 20°C and extending to about −80°C, for mitochondrial lipids from the roots of two ecotypes of tomato which respond differently to chilling at 5°C (2). Other workers (8) have interpreted these results as evidence to support their claim that sensitivity to chilling is not related to differences in the thermal behavior of membrane lipids.

Because of these reports the thermal response of the polar lipids from mitochondria of the chilling-sensitive plants soybean, mung bean, sweet potato, cucumber and tomato were examined using calorimetry and, for some preparations, spin labeling and ESR spectroscopy. Polar lipids from the mitochondria of the chilling-insensitive plants, wheat, barley, and artichoke were also examined by calorimetry. The results show that for each of the chilling-sensitive plants the membrane lipids undergo a phase transition at a temperature which correlates with the reported sensitivity and physiological response of the plant to chilling. No transition was detected in the lipids of the chilling-insensitive plants. The results also show that the transition temperature detected by spin labeling correlates with the initiation of the exothermic transition detected by calorimetry.

MATERIALS AND METHODS

Plants. Mung bean (Vigna radiata var Bergen) and soybean (Glycine max cv Davis) were grown for 4 d in the dark at 28°C. Tomato plants (Lycopersicon esculentum cv Flora-Dade and cv Grosse Lisse) were grown in a glasshouse and the fruit picked at the 'breaker' stage. Barley (Hordeum vulgare) and wheat (Triticum aestivum L. cv Falcon) were grown at 22°C for 4 d in the dark. Artichoke (Helianthus tuberosus L.) tubers were from plants grown in the field, washed and stored at 4°C as previously described by (1). Cucumber (Cucumis sativus L.) fruit and sweet potato (Ipomoea batatas L.) roots were obtained from commercial growers.

Mitochondria. Mitochondria were isolated from the hypocotyl of mung bean and soybean from the roots of barley and wheat
as described by Raison and Chapman (17), and from tomato, cucumber fruit, sweet potato roots, and dormant artichoke tubers as described by Raison and Lyons (20). Soybean seed was ground to a fine meal in a hammer mill.

**Lipid Extraction and Analysis.** Mitochondria and meal from soybean seed were extracted three times with CHCl3/CH3OH, 2/1 (v/v), as described in Method A of Fishwick and Wright (5) and washed with 0.55 m KCl and water as described by Raison and Wright (24). The polar lipids were separated from the neutral lipids by column chromatography on silica gel as described by Raison and Wright (24). The polar lipids of soybean seed and of mitochondria from hypocotyl tissue were separated by HPLC using a semipreparative, μ-Porasil column (Millipore Waters Inc.). The column was eluted using a linear gradient starting with hexane:iso-propanol-water, 6:8:0.4 (v/v/v) and changing to 6:8:1.2 (v/v/v) over 30 min at a flow rate of 3 ml min⁻¹. The components of each fraction were identified by TLC and the phospholipids quantitatively estimated by phosphorus analysis as described by Vaskovsky et al. (26).

**Electron Spin Resonance Spectroscopy.** Polar lipids from the mitochondria were suspended in 10 mm Tris/acetate buffer, pH 7.2, containing 0.5 mm EDTA by sonication. The spin label, dissolved in CH3OH, was deposited on the inside of a glass vial by evaporation. A portion of the lipid suspension was added and mixed with the spin label to give a molar ratio of spin label to lipid of 1:150. The spin label used was 3-oxazoladinyloxy-2-(10-carbethoxydeyl)-2-hexyl-4,4-dimethyl (12-nitroxide methylstearate, or 12NS).

**Calorimetry.** Thermograms were obtained using a differential scanning calorimeter (model DSC-2, Perkin-Elmer Corp., Norwalk, CT). Polar lipids dissolved in CHCl₃ were added to stainless steel pans (70 μl). The solvent was evaporated with a stream of N₂ and removed completely under vacuum. Buffer (20 mm Tris/acetate, pH 7.2, containing 2 mm EDTA) was added to give a 200% v/w excess, the pans were sealed and equilibrated at 37°C for 16 h. Scans were performed at either 2.5, 5, or 10°C deg min⁻¹ against a reference sample containing buffer and 3 to 6 mg of Sephadex to provide a similar thermal mass as the pan containing the lipid sample. The thermograms presented have been corrected for a baseline obtained using sample pans containing Sephadex and buffer. The temperature scale of the instrument was calibrated using an aqueous dispersion of dimyristoylphosphatidylcholine, melting point 23°C.

**RESULTS**

The thermal response of the polar lipids from soybean is shown in Figure 1. For the lipids of mitochondria from hypocotyl tissue the thermogram (Fig. 1, scan A) shows a sharp exotherm at 15°C and a more obtuse exotherm at about 3°C. From the assumed extension of the baseline shown (Fig. 1, scan A), it can be calculated that 9.6 mJ were evolved between 15°C and 0°C. If the exotherm represents the enthalpy due to a liquid crystalline to gel transition with an average of 25 kJ mol⁻¹ for the plant lipids (see Raison and Wright [24]), the data indicate that about 3% of the lipid is involved in the transition down to 0°C. For the polar lipids obtained from the total lipid extract of soybean seeds the exotherm shown in Figure 1, scan B, was not as sharp as that of the mitochondrial lipids but was evident at about 18°C with a more rapid increase in heat evolution at about 8°C and 3°C. The more obtuse exotherm for the polar lipids from seed, compared with that of mitochondria, is most likely a consequence of the more heterogeneous lipid component of the seed polar lipids (see Fig. 3).

It has been proposed that calorimetric transitions detected in biological systems at scan rates in excess of 5°C deg min⁻¹ could be artifacts resulting from thermal imbalance (25). As shown in Figure 2 the exotherm, detected in the soybean lipids at a scan rate of 10°C deg min⁻¹ (Fig. 2, scan A) was also detected at a scan rate of 5°C (Fig. 2, scan B) and 2.5°C deg min⁻¹ (Fig. 2, scan C) showing that the transitions observed were not anomalous features of the scan rate. The exotherm obtained at the slower scan rates commenced a few degrees higher than that observed at 10°C deg min⁻¹.

The polar lipids obtained from soybean seed and mitochondria from soybean hypocotyl were separated by HPLC and the components of each peak determined by TLC. As shown in Figure 3, the major phospholipids present in both samples were PC, PE, and PI. Smaller amounts of PS, CL, and PG were present in the lipids from seed and the proportion of each phospholipid was similar to that found by Harwood (6). The mitochondria from soybean hypocotyl contained, in addition to PC, PE, and PI, relatively large amounts of CL, typical of plant mitochondria (3).

**Thermograms of polar lipids from the mitochondria of chilling-sensitive and chilling-insensitive plants are shown in Figure 4.** For cucumber fruit, mung bean, and sweet potato, chilling-sensitive plants, an exotherm was initiated at about 15°C (Fig. 4A). In contrast, for barley, wheat, and artichoke, chilling-insensitive plants, no transition was evident in the temperature range studied (Fig. 4B).

The thermal response of the polar lipids of tomato fruit mitochondria is shown in Figure 5. For cv Grosse Lisse the exotherm was extremely sharp and occurred at 16°C and at 16.5°C for scan rates of 10 C and 5°C deg min⁻¹, respectively (Fig. 5A). For cv Flora-Dade the transition was initiated at 14°C (Fig. 5, scan C). As shown in Figure 5, scan D, the endothermic transition for the lipids from cv Grosse Lisse is complete by
The mung plants phase separated below 15°C for the temperature coefficient logarithm of the absolute motion of the transition is reversible. The hysteresis about 19°C, showing the transition is reversible. The hysteresis of about 5°C deg shown by the endotherm is similar to that found with binary mixtures of phospholipids at comparable scan rates (7).

The temperature dependent changes in ordering of the polar lipids from Grosse Lisse and mung bean were also investigated using ESR spectroscopy. The results are shown in Figure 6 where the logarithm of the motion parameter, τ0, is plotted against the reciprocal of the absolute temperature (°K⁻¹). As shown, the temperature coefficient of motion (the slope of the line) increases below 15°C for the tomato lipids (Fig. 6A) and below 12°C for the mung bean lipids (Fig. 6B) indicating that the lipids of these plants phase separated below the temperature of the transition detected by calorimetry.

**DISCUSSION**

The exotherm detected in the polar lipids of soybean seed and soybean mitochondria (Fig. 1) provide clear evidence that a phase transition occurs in these lipids within the temperature range associated with chilling injury. From calculations based on the heat evolved in the transition to 0°C it is estimated that about 2% of the seed polar lipid and about 3% of the mitochondrial lipids are in a gel phase at 0°C, indicating that only a small proportion of lipid, with a relatively high melting point, initiates, and is involved in, the transition down to 0°C. This is consistent with the estimates of the amount of lipid found to be involved in the transition of chloroplasts polar lipids of chilling-sensitive plants (24). The bulk of the membrane lipids of mitochondria are rich in unsaturated fatty acids (3) and thus would have relatively low melting points. These lipids would not be expected to solidify at temperatures above 0°C. This was the rationale used by O'Neill and Leopold (14) to support their inability to detect a transition in soybean polar lipids at chilling temperatures. However, since only a small proportion of membrane lipid appears to be involved in the transition a relationship between the temperature of the transition and the proportion of unsaturated fatty acids in the bulk of the membrane polar lipids would
not be expected. It is more likely that the transition temperature is related to the proportion of some particular lipid of relatively high melting-point, like DPPC as observed in chloroplast membranes (24) or to the proportion of DPPG plus 1-palmitoyl-2-(trans-Δ5-hexadecenoyl)PG as proposed by Murata (12).

It should be stressed that the detection of an exotherm in the membrane polar lipids of chilling-sensitive species does not unequivocally establish that the lipids have undergone a phase change from a liquid crystalline to gel state. It is possible that some, or all, of the heat detected is derived from the entropy of mixing due to phase separation (22). If some of the heat is derived from this source then the transition observed would involve even less lipid than indicated by the calculations above. However, regardless of which physical description is applied, the results demonstrate that a transition occurs in the membrane lipids of mitochondria from soybean at a temperature which relates to a change in the physiological response of these mitochondria (4) and the temperature below which the plant suffers visible symptoms of chilling injury (13). The results presented are, however, in sharp contrast with those reported by O’Neill and Leopold (14) who were unable to detect a transition in the lipids from soybean seed or from mitochondria from hypocotyl tissue. It is difficult to explain the discrepancy in the two results. It is not clear if the polar lipids from soybean seed, used by O’Neill and Leopold (14), were separated from the total lipid extract by chromatography on acid-washed Florisil or by precipitation with acetone. Both methods were described and reported to give ‘essentially’ the same results but only the results obtained using the precipitated lipids were shown. Since no analysis was given it is not possible to determine if precipitation with acetone quantitatively separated all of the phospholipids nor is it possible to compare the efficiency of the two procedures used by O’Neill and Leopold (14). In contrast, the polar lipids extracted from soybean seed by the methods described in this paper (Fig. 3) were quantitatively similar to the phospholipids previously reported present in the polar lipid fraction of soybean seed by Harwood (6). It is therefore concluded that the transition detected in the seed polar lipids (Fig. 2) is a realistic reflection of their thermal behavior. Furthermore, the composition of the polar lipids from the mitochondria of hypocotyl tissue are typical of plant mitochondria (3) and the relatively abrupt exotherm displayed by these lipids, compared with the seed polar lipids (Fig. 1), is consistent with the absence of low melting point, galactolipids (Fig. 3) in the mitochondrial lipids.

While it is difficult to determine a precise temperature below which chilling develops in fruits and tissue it is clear that the temperature of the transition in the polar lipids of mitochondria from mung bean hypocotyl, cucumber fruit, and sweet potato roots is only a few degrees higher than the critical temperature reported for these tissues. For mung bean the transition is at 14°C and growth of young plants declines rapidly below 15°C (17). Similarly for cucumber and sweet potato, the transition is at 15°C (Fig. 4) and they suffer visible injury when stored for 14 d at 10°C (11). Sweet potato can be stored for 6 months at 15°C (11) indicating that the critical temperature for this species is between 10°C and 15°C. The absence of an exotherm in the polar lipids from mitochondria of the chilling-insensitive plants is also consistent with the insensitivity of these species to chilling.

A phase transition was also observed in the lipids of mitochondria from both cultivars of tomato and the temperature is only a few degrees higher than that below which chilling injury becomes evident in tomato fruit (11). The temperature coefficient of motion for the spin label intercalated with tomato polar lipids also increased below the temperature of the transition detected by calorimetry, showing that the polar lipids phase separated below this temperature. The temperature of the exotherm for the varieties of tomatoes investigated was a few degrees lower than that observed by Dalziel and Breidenbach (2) for the polar lipids of mitochondria from root tissue of two ecotypes of L. hirsutum studied. These authors did not detect phase separation using a spin label derivative of tetradeacne. However, as the authors point out, this label may not preferentially partition into the gel phase and therefore might only detect the completion of a transition (2).

The calorimetric data reported by Dalziel and Breidenbach (2), and those reported here are in general agreement. Dalziel and Breidenbach (2), however, made the point that, while the thermal response of the lipids from the two ecotypes was similar, the response of the tomato plants to chilling differed. They thus concluded that the response to chilling could not be attributed to the physical changes induced in the mitochondrial membrane lipids. This conclusion, however, is predicated on the assumption that the two ecotypes studied vary in their sensitivity to chilling. In the experiment described by Dalziel and Breidenbach (2) the two plants were exposed to 5°C and the time for death of the plants noted. This method of assessing chilling injury measured the time course for development of injury of plants exposed to 5°C rather than the temperature below which injury becomes evident. The plants from both ecotypes died, those from the high altitude requiring 5 d compared with 1 d for those from the low altitude regions. Thus both were susceptible to chilling injury at 5°C and in this regard their physiological response to exposure to 5°C was consistent with a phase change in the membrane lipids at 16°C. It is important to consider that a manifestation of chilling injury at the tissue level, or the death of a plant exposed to a chilling temperature, is the end result of a complex series of events which are dependent on many factors. This means that while the events which initiate injury might occur at the same temperature in the two ecotypes, the time for some measured response to become evident could differ. It is the response to chilling that was measured by Dalziel and Breidenbach (2). They did not determine the temperature, previously defined as the critical temperature (16, 17), which determines the sensitivity of a plant to chilling. It is the events initiated at this temperature which correlate with the temperature of the phase transition in the membrane lipids (9, 19, 23). Thus it is possible that the two ecotypes of tomato studied by Dalziel and Breidenbach (2) display the same sensitivity to chilling. Differences in the response of the two ecotypes when exposed to 5°C are most likely manifestations of differences in the rates of any one of a multitude of secondary events (16) associated with chilling injury. Differences in the time response of these secondary events are not directly relevant to the hypothesis linking the primary event.
of chilling injury with a phase change in the membrane lipids.

Collectively, the results reported here show that for a number of chilling-sensitive plants a phase transition occurs in the polar lipids of mitochondria at a temperature which correlates with the sensitivity of the plant to chilling injury. These transitions are not observed in lipids from chilling-insensitive plants. The results also refute the arguments outlined by O’Neill and Leopold (14) suggesting that chilling injury is not induced by a phase change in membrane lipids. Furthermore, the paper points out the necessity of clearly differentiating between experiments designed to measure the time response of a plant or tissue to chilling and those designed to measure the sensitivity to chilling where sensitivity is defined in terms of a temperature below which injury develops.

LITERATURE CITED