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QUICK-FREEZE DIFFERENTIAL SCANNING CALORIMETRY AND SATURATION TRANSFER ELECTRON SPIN RESONANCE: NOVEL TECHNIQUES FOR ASSESSING PHASE TRANSITIONS IN BIOLOGICAL MEMBRANES

Alejandro G. Marangoni and David W. Stanley

University of Guelph, Department of Food Science
Guelph, Ontario N1G 2W1, Canada

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Quick-freeze differential scanning calorimetry (QF-DSC) and saturation transfer-electron spin resonance (ST-ESR) spectroscopy were used to study lipid gel-phase transitions in mature green tomato fruit microsomal membranes. ST-ESR of 12-doxyl methyl stearate labelled membranes proved to be reproducible and provided increased sensitivity to temperature-induced structural changes, allowing the detection of several transitions in isolated membranes (6°C, 21°C, 28°C). QF-DSC led to the assessment of lipid gel phase transitions in isolated microsomal membranes and microsomal membrane lipids by enhancing the transition. A phase transition enthalpy of 114 J/g and an onset temperature of 29.8°C were obtained for whole membranes while with isolated lipids values of 370 J/g and 19.9°C were found.

Membrane rigidification and phase changes have been associated with senescence, chilling injury and dehydration damage in plants (1,2,3). Electron spin resonance (ESR) and differential scanning calorimetry (DSC) have been two of the main tools used to study these processes. DSC has been used to assess phase transitions in isolated phospholipids from mitochondria and thylakoids (4,5). DSC, however, has not proven successful in assessing phase transitions in whole membranes. The main difficulty arises from high sterol contents and probable lipid-lipid and protein-lipid associations which affect the phase behaviour of biological membranes (6,7,8).

ESR has been used to assess phase transitions in isolated phospholipids from mitochondria and thylakoids (4,5) and recently in isolated microsomes (9). Generally, rotational correlation times (RCT) are calculated from ESR spectra assuming isotropic or pseudo-isotropic motion of the spin probe, and plotted as Arrhenius plots. "Breaks" (if present) in these plots are interpreted as phase transitions. Rotational correlation times of spin probes in biological membranes are in the order of nanoseconds. We have found that spectra obtained from probes in this time range are generally quite insensitive to temperature

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changes, thus determination of RCT then becomes even more subjective. Saturation transfer - ESR (ST=ESR) uses the second derivative, 90° out of phase signal from the spin probe, as opposed to the usual first derivative, in phase, signal. ST-ESR is used in RCT ranges of 10^{-8} sec to 10^{-3} sec (10). We have found that ST-ESR in this "non ST-ESR" range was much more sensitive to temperature changes than the usual first derivative ESR spectrum. RCT can be determined from a theoretically derived function relating different peak height ratios to the RCT (10), or by making a "standard curve" (see Discussion).

Quick-freeze differential scanning calorimetry (QF-DSC) was first used by Melchior (11) to separate transitions of a mixture of dimyristoyl and distearyl phosphatidylcholine liposomes, proving fluid-lipid immiscibility in this artificial mixture. This author suggested that quick freezing membranes or hydrated lipids from 85°C to -196°C would prevent the "mixing" of membrane lipid domains, thus achieving the crystallization of relatively pure domains within the membrane. The theoretical basis for the existence of lipid domains in crystalline membranes has been presented elsewhere (11). The efficacy of the method depends on the freezing rate of the coolant being faster than the lateral diffusion of membrane lipids and the lateral motion of the lipids in the crystalline state must be slow enough to allow measurement before the system relaxes back to the equilibrium state (11). In this communication we report the use of ST-ESR and QF-DSC in the assessment of phase transitions in tomato fruit microsomal membranes.

METHODOLOGY

Plants. Tomato plants (Lycopersicon esculentum var. Caruso) were grown in standard greenhouse conditions. Plants were fertilized bi-weekly with 20-20-20 NPK and calcium nitrate. Mature green fruit stages 2 to 4 were selected according to established criteria (12).

Microsomal isolation. Microsomes were isolated as described previously (9) for the DSC and ESR studies respectively.

Spin labelling and ST-ESR measurements. Spin labelling was performed as described before (9). The probe used was 2-hexyl-2-[11-methoxy-11-oxoundecyl]-4,4-dimethyl-3-oxazolidinyloxy, or 12 doxyl stearic acid methyl ester (Sigma). The saturation transfer condition was obtained as follows: using a stable free radical containing solid, diphenyl-picryl-hydrazyl, the maximum second derivative, in phase, signal was obtained by adjusting the phase knob at a low power setting - 20 mW. Once this condition was obtained the signal was switched to 90° out of phase giving a total of 105.2° out of phase considering the former adjustments. The labelled membrane was loaded and the power increased to 100 mW. Modulation amplitude was set at 5 G, modulation frequency at 100 kHz, time constant at 100 msec and scan time at 50 sec. Ten spectra were accumulated at each temperature. Points were taken every 4°C from 0 to 44°C. All measurements were performed in a Bruker ER 200 D - SRC spectrometer (Bruker Analytische Messtechnik GHBH). Values of C´/C (see Fig. 1) were plotted as a function of temperature.

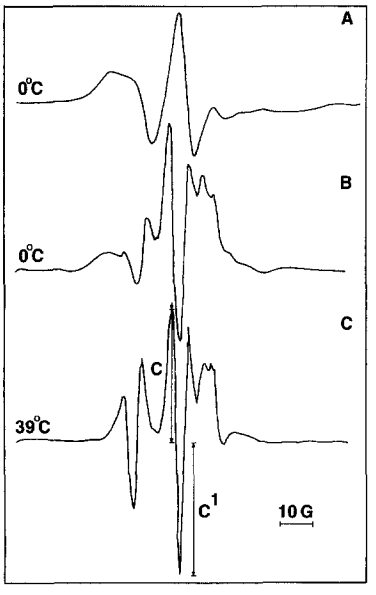
Quick-freeze DSC. A 15 mg sample of wet microsomal pellet was placed in an aluminum DSC pan and sealed. The pan was equilibrated at 85°C for 15 min and quickly dropped into liquid Freon 22 held at liquid nitrogen temperatures. The freezing rate of Freon 22 is reported as 66,000°C/sec. The temperature in the calorimeter cell was lowered to -170°C with liquid nitrogen. The sample was then quickly transferred to the cell. Temperature was raised quickly to

-30°C and samples were run from -30°C to +70°C at a heating rate of 8°C/min. After each run pans were pierced and dried at 150°C for 12 h in order to determine the dry weight of the membranes for enthalpy calculations.

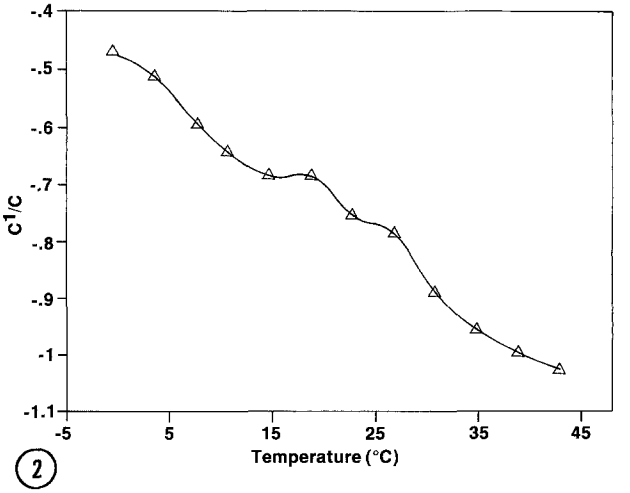
Membrane lipids were extracted as described previously (9). Approximately 1 mg of lipid dissolved in $CHCl_3$ were transferred to a DSC pan and the solvent was evaporated under a gentle stream of nitrogen. Lipids were overlayed with 10 µl of buffer, pans sealed and run as described above. All DSC runs and data analysis were performed in a DuPont 1090 thermal analyzer.

RESULTS AND DISCUSSION

ST-ESR spectra of the spin labelled microsomal membranes at 0°C and 39°C are shown in Figures 1b and 1c. A first derivative ESR spectrum at 0°C is shown in Fig. 1a. A plot of C´/C as a function of temperature (Fig. 2) shows phase transitions at about 21°C with other minor transitions at 6°C and 28°C. Temperatures of transition occurred at inflection points in those ranges showing the largest proportional change in C´/C. Although changes were subtle, the determination of points of inflection is decidedly objective. In order to determine the rotational correlation time for the probe, two procedures could be used. The first is to use a theoretically derived function which relates RCT to different peak ratios from the spectra (10). It was found that only the C´/C function could be used because the signal obtained is too fast, that is, RCT is shorter than 10^{-8} sec. On the other hand, if the C´/C function is used,



(1)



(2)

Figure 1. ST-ESR spectra at 0°C (B) and 39°C (C) and first derivative ESR spectrum at 0°C (A) for 12 NS spin labelled tomato microsomal membranes.

Figure 2. Peak height ratios C^1/C (see Figure 1) as a function of temperature. Transition temperatures are seen at 6, 21 and 28°C.

RCTs in the order of 10^{-5} to 10^{-8} sec are found which is too low. The second way of determining RCT would be by dissolving the probe in a solution that matches the viscosity of the medium in which the probe is dissolved (e.g., the membrane). A temperature run could then be performed and a standard curve derived, relating temperature to RCT, calculated as previously described (5). Attempts were made using glycerol-water mixtures but it was found that the spin probe was insoluble in this system.

Fig. 3a shows the QF-DSC thermogram for the isolated microsomal membranes from tomato fruit relative to the water melting endotherm. Fig. 3c shows the expanded version of the former transition while Fig. 3d shows the phase transition of the isolated lipids from the same membrane preparation. Thermodynamic parameters for the phase transitions are presented in Table 1. The transition temperature for the extracted lipids is lower than that of the membranes (19.9°C vs 29.8°C) but corresponds to the major transition determined by ST-ESR (21°C) for the spin labelled membrane. This suggests that the phase transition observed by ST-ESR corresponds to that of the membrane lipids. It is interesting to notice that the transitions are not evident unless the lipids and membranes are heated prior to freezing. This has been attributed to lipid-

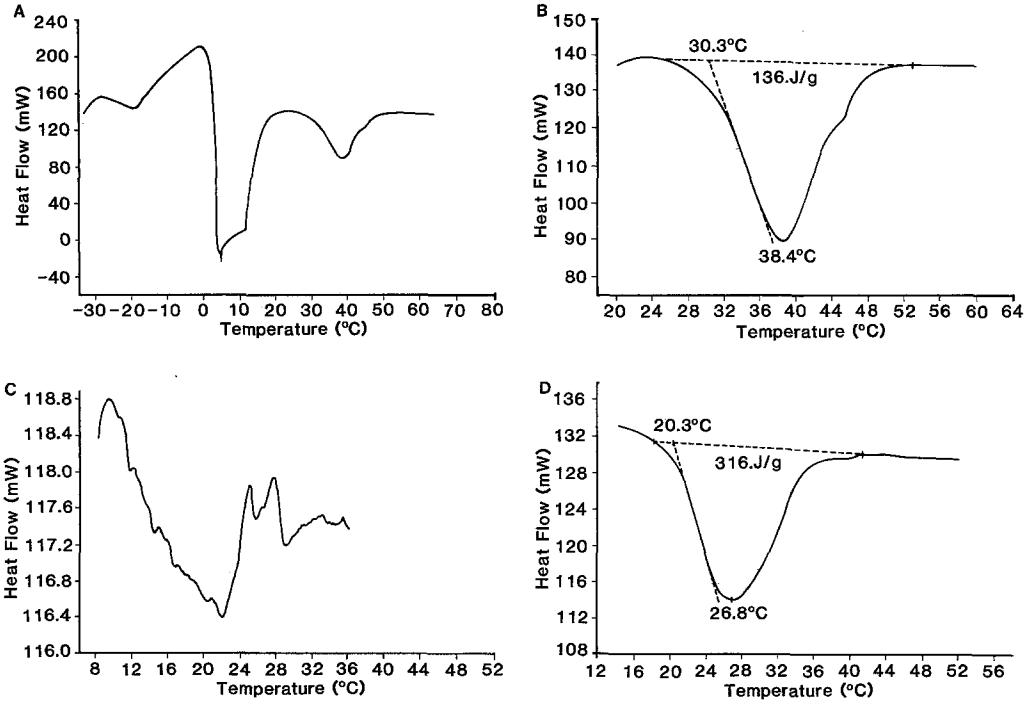


Figure 3. QF-DSC thermograms of isolated tomato fruit microsomal membranes run at 8°C/min (A) and 2°C/min (B). Larger endotherm (A) corresponds to melting of ice. Numerous lipid phase transitions can be appreciated (B) at slower heating rates corresponding to different membrane microenvironments differing in composition. Membrane phase transitions (C) occur at a higher temperature than membrane lipid phase transitions (D).

Table 1. Thermodynamic data derived from quick-freeze differential scanning calorimetric studies of lipid phase transitions in tomato microsomal membranes and membrane lipids. Values shown are means (standard deviation) of at least three independent membrane preparations. Enthalpies are expressed per gram of dry membrane and per gram of isolated lipid.

PARAMETER	MEMBRANES	LIPIDS
Onset Temperature (°C)	29.8 (0.70)	19.9 (1.50)
Peak Temperature (°C)	37.4 (1.48)	26.6 (1.50)
Enthalpy (J/g)	114 (47)	370 (47)

lipid interactions in tomato fruit microsomes (6). Slow freezing of the heat denatured membranes produced a small endotherm in the same temperature range as the quick frozen samples (thermogram not shown), suggesting lipid domain mixing during freezing. When slower heating rates (Fig. 3b) were used, two opposing effects came into play: lipid domains began to mix, dampening out the transition, but resolution improved, allowing for the detection of lipid microenvironment melting transitions. Curve resolution methodology could be applied to study membrane microenvironments in this way. To our knowledge, this is the first report in which QF-DSC has been applied to the study of whole membranes. These two methods seem promising and are being currently applied in our laboratory to assess phase transitions in chill-injured membranes.

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