Temperature-induced Phase Changes in Mitochondrial Membranes Detected by Spin Labeling

J. K. Raison, J. M. Lyons, R. J. Mehlhorn, and A. D. Keith

From the Department of Vegetable Crops, University of California, Riverside, California 92502, and the Department of Genetics, University of California, Berkeley, California 94720

SUMMARY

Molecular motion data from a spin-labeled fatty acid presented on Arrhenius graphs allow melting points or phase transitions to be inferred in local domains such as membranes. With use of this technique, mitochondria from a chilling-sensitive plant and a homeothermic animal displayed temperature-dependent phase transitions at approximately 12° and 23°, respectively. These temperatures coincide identically with the temperatures below which chilling-sensitive plants (12°) or homeothermic animals (23°) are injured upon exposure. Mitochondria from a chilling-resistant plant and a poikilothermic animal did not display any such phase transitions. These data indicate that some membrane-bound enzymes are sensitive to the physical state of membrane lipids.

Previous studies (1, 2) have shown that the respiratory activity of mitochondria isolated from tissues of chilling-sensitive plants and homoeothermic animals exhibits a disproportionate decrease when the temperature is reduced below 10–12° and 23–24°, respectively. This abrupt change in respiratory activity was reflected in an Arrhenius plot as a change in activation energy, giving the appearance of a phase change, and in some cases appeared on the graph as a discontinuity at the transition temperature. In contrast, mitochondria from chilling-resistant plant tissues and the liver of poikilothermic animals exhibited a constant activation energy from 1–36°. Since the lipids of mitochondria from chilling-sensitive plants (3) and the liver of homoeothermic animals (4) contained a relatively greater proportion of saturated fatty acids than the resistant plants or poikilotherms, thermic animals (4) contained a relatively greater proportion of these components of mitochondrial membranes, which are injured upon exposure. Mitochondria from a chilling-resistant plant and a poikilothermic animal did not display any such phase transitions. These data indicate that some membrane-bound enzymes are sensitive to the physical state of membrane lipids.

kinetic properties of respiratory enzymes. Consequently, it is important to determine whether the temperature-induced phase change in the membrane is an intrinsic property of the lipid, the protein, or an association of lipid-protein components within the membrane structure.

Recent applications of the use of spin-labeled compounds to determine the physical state of lipid components in membranes provide a direct physical method of elucidating more about the physical nature of this temperature-induced transition. One of these applications describes the effect of temperature on the mobility of a spin-labeled compound associated with the lipid components of Mycoplasma laidlawii (6) displayed on an Arrhenius plot. Molecular motion data presented in this way can be used to infer melting points or physical transitions in lipid systems. Through use of these techniques, the results presented here show that the lipid components of the mitochondria from chilling-sensitive plants and rat liver undergo thermal phase transitions at approximately 12° and 23°, respectively. Phase transitions were observed at the same temperatures when mitochondria were heated to 60° and were then spin labeled and when lipids were extracted from mitochondria. No phase change was observed with mitochondria or the lipids extracted from chilling-resistant plants or fish liver. The results indicate that the temperature-induced phase change in temperature-sensitive mitochondrial membranes is an intrinsic property of the membrane lipids and that a direct correlation between the physical state of membrane components and enzyme activity can be established.

METHODS

Mitochondria were isolated from sweet potato roots (Ipomoea batatas L.), potato tubers (Solanum tuberosum L.), rat liver (Sprague-Dawley Albino), and rainbow trout liver (Ictalurus punctatus) by the methods described previously (1, 2). All operations were performed below 4°, and the isolated mitochondria were stored in ice. Electron spin resonance spectra were recorded with a Varian V-4500 X-band spectrometer. The temperature of the sample was varied between 1° and 40° with a Varian variable temperature control unit calibrated with an iron-constantan thermocouple and maintained at an estimated accuracy of ±1.5°.

A Folch (7) extraction and wash was carried out on sweet potato and rat liver mitochondria. These extracts were dried

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under vacuum, streaked on preparative thin layer chromatography plates (Silica Gel G), developed with diethyl ether to move neutral lipids to the front (the top 3 cm were scraped off), and developed in chloroform-methanol-water (65:25:4). The space between, 1 cm above the origin and 2 cm below the front, was scraped off and exhaustively eluted with anhydrous methanol. This extract contained most of the phospholipids, but the distribution of phospholipid classes was not further analyzed. This extract was sonicated into 0.1 M Tris buffer (pH 7.2) at a concentration of 10 mg per ml, and 12NS² (see Structure I) was then added in the same manner as to mitochondrial preparations. The dispersions were analyzed directly by electron spin resonance.

The spin-labeled fatty acid (12NS) was synthesized by the method of Keams, Keana, and Beetham (8) and is described in detail elsewhere (9). The methyl ester of 12NS was used in all preparations. Another spin label, 4-amino-(4N-myristate)-2,2,6,6-tetramethylpiperidine-1N oxyl (TAM), was synthesized by treating myristyl chloride with 4-amino-2,2,6,6-tetramethylpiperidine-1N oxyl in pyridine, by means of textbook procedures for synthesizing acyl amines. TAM (see Structure II) was purified by preparative thin layer chromatography to a homogeneous band with chloroform on Silica Gel G. The spin labels (dissolved in ethanol at 10⁻⁰₅ M) were added to approximately 0.2 ml of mitochondrial suspension (1.5 mg to 5 mg of protein) to make an approximate bulk concentration of 2 × 10⁻⁴ M spin label, and the spectra were recorded immediately.

Stearic acid over 99% pure was obtained from the Hormel Institute, Austin, Minnesota, and was not further analyzed. A less pure stearic acid obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, was analyzed by gas-liquid chromatography and found to be 97 ± 1% pure.

RESULTS

In the low viscosity limit the rotational correlation time (τᵣ) of the spin label can be obtained from the expression

\[ \tauᵣ = K(W₁ - W₄) \]  (1)

where \( W₃ \) and \( W₁ \) are widths of the mid- and high field lines on a first derivative absorption spectrum. Formula 1 can be derived from Kivelson (10) and is similar to expressions used by other researchers in the last few years. While \( τᵣ \) can be defined mathematically in terms of an isotropic rotational diffusion rate, its physical meaning on the molecular level is not precise. Roughly speaking, it is the time required for a molecule to tumble through an arc of 90°. \( K \) is a constant which depends on the anisotropic hyperfine coupling (\( Aₓ \)) values and the anisotropic g values. The spectral values of Griffith, Cornell, and McConnell (11) were used to calculate \( K = 6.5 \times 10⁻¹⁹ \) s. Expressions for \( τᵣ \) such as the one presented here are valid for isotropic motion, which would be expected to originate only from spherical molecules. 12NS is far from spherical and might be expected to exhibit enhanced averaging of \( x - y \) anisotropy; however, studies with a variety of spin-labeled fatty acids have shown that the departure from isotropic tumbling is related to the proximity of the nitrogen atom to the carboxyl group of the fatty acid (12). It was also shown that 12NS moves in a nearly isotropic fashion, so our use of Kivelson's formula is warranted. By assuming Lorentzian line shapes, the data reduction can be greatly facilitated by writing \( W₁/W₄ = \sqrt{h₁/h₋₁} \), where \( h₁ \) refers to the first derivative line height. For correlation times \( τᵣ \gtrsim 10⁻⁸ \) s, \( K \) becomes dependent on the motion of the nitroxide molecule. Spectra in this tumbling range can, nevertheless, be analyzed in terms of an empirical motion parameter

\[ τᵣ = 6.5 \times 10⁻¹⁹ W₃ \left( \frac{\sqrt{h₁/h₋₁} - 1}{s} \right) \]

 wherever line heights and widths are measurable. Definition 2 was used to analyze the motion of selected spin labels in mitochondria over a broad temperature range. Apart from having a defined theoretical basis in the fast tumbling region, this expression has the advantage that broadening interactions which affect all lines equally are substantially subtracted out.

General Environmental Considerations—A series of spectra for 12NS in stearic acid at different temperatures illustrating changes in line shape at different states of motion is shown in Fig. 1. The spectrum at 100°C reflects an environment of low viscosity characterized by three narrow lines. The spectrum at -100°C shows a broadened spectrum characteristic of environments of high viscosity where the motion of 12NS is restricted. The other spectra illustrate the gradual change from free to restricted motion. The spectra of the mitochondria and phospholipid preparations fall between these extremes. Rat liver mitochondrial phospholipids at 22°C are shown as a representative spectrum. Spectra such as that of stearic acid at -20°C are too broad for \( τᵣ \) line measurements to be carried out. The stearic acid spectra at 60° and 0°C illustrate the spectral measurements made for the present work. All six preparations dealt with here (mitochondria from sweet potato, potato, fish, and rat liver and phospholipids extracted from sweet potato and rat liver mitochondria) have the same general state of fluidity. At 20°C \( τᵣ \) varied among the preparations by a factor of at most three. All spectra shown in Fig. 1 can be mimicked by varying the temperature of a glycerol-12NS mixture between 50° and 70°C.

Another environmental factor to consider is the hyperfine coupling constant (\( Aₓ \)). The \( Aₓ \) value for an oxazolidine nitroxide, such as 12NS, attached to a dipolar group varies from about 14.2 gauss in hydrocarbon solvents to about 16.1 gauss in water at room temperature. 12NS has an \( Aₓ \) equal to 14.2 gauss in octadecane at 30°C, and 15.7 gauss in 20% aqueous ethanol at 20°C. The \( Aₓ \) for 12NS in all mitochondrial preparations at 50°C was 14.2 to 14.3 gauss and, in the two phospholipid preparations, 14.3 to 14.4 gauss. These values indicate that 12NS was in nonpolar zones in the mitochondrial and phospholipid dispersions, with less water exclusion in the phospholipid dispersions.

The spin-labeled TAM added to rat liver mitochondria lost its electron spin resonance signal at 20°C in about 10 to 15 min.
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12 NS in 18:0 at 100°C
$T_c = 1.4 \times 10^{-10} \text{sec}^{-1}$

12 NS in 18:0 at 60°C
$T_c = 3 \times 10^{-10} \text{sec}^{-1}$

12 NS in 18:0 at 0°C
$T_c = 2.4 \times 10^{-10} \text{sec}^{-1}$

12 NS in 18:0 at 20°C

12 NS in 18:0 at -20°C

12 NS in 18:0 at -50°C

12 NS in Rat liver mitochondrial phospholipids at 22°C
$T_c = 2.6 \times 10^{-10} \text{sec}^{-1}$

Fig. 1. Spectra of 12NS in 97% purity stearic acid (the spectra exchange broadens in 99+% stearate below about 40°C) are shown at several different temperatures. As a representative spectrum of the biological samples, 12NS in rat liver phospholipids is shown at 22°C. The spectrum from the phospholipid example is at a state of motion similar to that of 12NS in stearate at 0°C. These two spectra appear somewhat different, due to a slightly different X-axis scale (refer to gauss markers, G).

TAM added to mitochondria from sweet potato and potato did not show detectable loss of signal over the same period. 12NS in rat liver mitochondria also suffered loss of signal at a much slower rate. Above 35°C destruction was detectable and at 40°C half the signal disappeared in 10 to 20 min; however, this varies among different preparations of mitochondria from the same organism and sometimes may be outside these time intervals. Fish liver mitochondria resulted in destruction of the 12NS signal at a slower rate. For the period required to perform the analysis (about 4 hours) the phospholipid dispersions gave no loss of signal at 40°C and below.

Arrhenius Activation Energies and Phase Changes—For a given preparation, phase changes can be deduced by displaying correlation times on an Arrhenius plot. An example of this technique for a model system may be seen in Fig. 2, which shows that 12NS in stearic acid is characterized by activation energies which depend on the phase of the solvent, having a different $E_a$ above and below the optical melting point (70°C). No attempt is made

Fig. 2. $\tau_c$ of 12NS in stearic acid (greater than 99% purity) is shown on an Arrhenius plot over a temperature range illustrating the change in $E_a$ at the observed (optical) melting point.

Fig. 3. Arrhenius plots are shown for the different mitochondrial preparations employed. $E_a$ values are shown above each plot.

Fig. 4. Arrhenius plots are shown for the different mitochondrial preparations employed. $E_a$ values are shown above each plot.
Fatty acid composition. The extracted lipids of mycoplasma and plasma cells, membranes, and polar lipid extracts (14-16) demonstrate a nonlinearity in the measurements as a function of temperature. Thermal analysis (DSC or DTA) has a long industrial history and has been used recently on a number of systems. The temperature-dependent data in Fig. 3 show a straight line for the motion of 12NS in mitochondria of fish liver and potato, the poikilothermic animal, and the chilling-resistant plant. Data plotted in the same way for mitochondria from sweet potato and rat liver show perturbations at the previously established critical temperature for each, about 12°C for the chilling-sensitive plant and 29°C for the homeothermic animal. Mitochondria which were heat denatured from both sweet potato and rat liver (60°C for 15 min) and then spin labeled showed the same temperatures of $E_0$ change as before. The motion at a given temperature was more free than before denaturation. Phospholipids extracted (Fig. 4) from mitochondria of sweet potato and rat liver show changes in activation energy at the same temperatures as do the mitochondrial sources (about 12°C and 23°C).

**Discussion**

A number of physical methods have recently been used to demonstrate or suggest physical phase transitions in lipids. The examples given below exploit some physical measurement to demonstrate a nonlinearity in the measurements as a function of temperature. Thermal analysis (DSC or DTA) has a long industrial history and has been used recently on a number of natural product lipids (cf. Reference 13, for review). Mycoplasma cells, membranes, and polar lipid extracts (14-16) demonstrate thermal phase transitions which vary as a function of the fatty acid composition. The extracted lipids of mycoplasma and membrane preparations having the same fatty acid composition show transitions at the same temperatures. Phospholipid vesicles have been shown to exhibit temperature-dependent permeability effects which relate to the fatty acid composition (17), therefore, making it possible to relate these findings to "melts" in the hydrocarbon zones of phospholipids. The temperature dependence of film expansion in *Escherichia coli* phosphatidyl ethanolamine having different fatty acids compositions correlated reasonably well with the temperature dependence of β-galactoside transport into *E. coli* cells having approximately the same fatty acid composition as was used in the film expansion studies (18). Polarization microscopy has been used to infer phase changes in lipid systems (19). Another physical technique of observing phase transitions exploits x-ray diffraction. Engelmann (20) has observed lipid melts both in fatty acid-supplemented mycoplasma cells and their isolated membranes. The melting points, as reflected in low and high angle spacing changes, correlated with the fatty acid enrichment. As with DSC, this technique allows the range of a melt to be inferred. Transitions were seen to occur over a range of 6-10°C.

Since phospholipids exhibit several structural forms or morphic lyotrophisms as a function of hydration, temperature, fatty acid composition, polar head group(s), perturbing molecules and so on, a simple phenomenological nonlinearity presents difficulties in interpretation. Conversely, biological observations demonstrating nonlinear temperature dependencies such as those dealing with O2 uptake by isolated mitochondria (1, 2) or β-galactose transport in *E. coli* as a function of fatty acid composition (21, 22) are also difficult to interpret because of a lack of physical measurements and, consequently, are not well correlated with structure. The present report establishes a correspondence between the O2 uptake nonlinearity of Lyons and Raison (1, 2), organism resistance to cold exposure, and an electron spin resonance motion parameter, in the same systems. More simply stated, these observations relate physiological changes to physical structural changes and indicate physiological dependency on structure.

It is well known that the degree of unsaturation in organism lipids correlates with growth temperature. It is usually observed that the degree of unsaturation is inversely related to the growth temperature and this in turn is thought to aid in the maintenance of a relatively constant lipid or membrane lipid viscosity. For example, it has been generally observed that marine organisms show an increased degree of unsaturation as the average temperature of their habitat is reduced (23). For bacteria (24), fungi (25), plants (26), and insects (26) there exists a similar relationship, showing an inverse relation between degree of unsaturation and developmental temperature. The recent development of lipid mutants in yeast (27), *E. coli* (28), and *Neurospora* (29) offers promise in exploiting the control of fatty acid composition as a method of determining the functional role of lipids in membranes. For example, a fatty acid chain elongation mutant of *Neurospora* grows only when supplemented with a saturated fatty acid (29). During log phase growth, removal of the fatty acid supplement results in growth stoppage after about two divisions. Examination of this mutant's lipids with 12NS revealed a 2- to 3-fold increase in lipid viscosity in going from the supplemented growing state to the point where cell division stopped. In the present report, the potato mitochondria are more unsaturated than those of sweet potato, and trout liver mitochondria are more unsaturated than those of rat liver; however, all four systems have considerable fatty acid heterogeneity and it is not clear
that the ratio of unsaturated to saturated is the determining factor as to whether a phase transition occurs. In fact, it also seems reasonable that the acyl pattern of fatty acids and nitrogen bases on phospholipids may constitute another level of structural ordering.

Data presented here indicate that the general state of hydrocarbon fluidity is similar in mitochondria from sweet potato, rat liver, potato, and trout liver and is about the same as that reported for other membranes investigated with spin labels. The degree of viscosity is affected by the fatty acid composition of the particular membrane system, and a departure from linearity in a plot of log $\tau_0$ versus $^\circ$K$^{-1}$ appears to be a function of the mitochondrial lipids. However, at a given temperature the exact state of molecular motion in a membrane system may be quite different from that in its extracted phospholipids.

A perturbation in the rate of oxygen uptake as a function of temperature was displayed on an Arrhenius plot by sweet potato (1) and rat liver (2) mitochondria at the temperature where we observed similar perturbations in $\tau_0$ of 12NS mitochondria from the same sources. No such events in oxygen uptake or $\tau_0$ were observed in potato and trout liver mitochondria. The coincidence of oxygen uptake rate departing from linearity at the same temperatures in the two mitochondrial systems as $\tau_0$ of a spin label in the same mitochondrial systems, establishes a relationship. Since oxygen uptake is enzyme dependent, we think these results imply that the function of some membrane-bound enzymes is influenced by the physical state of membrane lipids.

This generalization may be extremely important in the understanding of resistance to chilling of plants, temperature tolerance of animals, and other physiological processes.

REFERENCES

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