O₂-triggered Changes of Membrane Fatty Acid Composition Have No Effect on Arrhenius Discontinuities of Respiration in Sycamore (Acer pseudoplatanus L.) Cells*

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Sycamore cells (Acer pseudoplatanus L.) in suspension culture were grown at 25 °C in culture medium containing two oxygen concentrations: 250 μM O₂ (standard conditions) and 10 μM O₂ (O₂-limiting conditions). The decrease of O₂ concentration in the culture medium did not modify significantly the relative proportion of each phospholipid. In contrast, the molar proportion of fatty acids was dramatically changed in all lipid classes of the cell membranes; the average percentage of oleate increased from 3 to 45% whereas that of linoleate decreased from 49 to 22%. When normal culture conditions were restored (250 μM O₂), oleate underwent a rapid desaturation process; the loss of oleic acid was associated with a stoichiometric appearance of linoleic acid at a rate of about 4 nmol of oleate desaturated/h/10⁶ cells. Under these conditions, no change in the Arrhenius-type plots of the rate of sycamore cell respiration was observed; the values of the transition temperature and of the Arrhenius activation energy (Eₐ) associated with the cell respiration as well as with the respiration-associated enzymes remained unchanged. Thus it was concluded that the fact that a strong decrease in the fraction of unsaturated fatty acid residues present in the mitochondria had no effect on electron transport rates and Arrhenius plot discontinuities casts doubt on the significance of such changes in terms of chilling injury. Finally it is suggested that some of the Arrhenius discontinuities observed at the level of membrane enzyme could be the consequence of intrinsic thermotropic changes in protein arrangement independent of lipid fluidity.

It has been frequently suggested that the composition of the fatty acid components of membrane phospholipids is temperature-dependent; fatty acids are normally more unsaturated in the membrane systems of chilling-resistant plants than in those of chilling-sensitive plants (1-5). Furthermore, authors suggested that there is a correlation between chilling sensitivity and the occurrence of characteristic discontinuities in the temperature-dependent activities of specific membrane enzymes and parameters reflecting the physiological state (gel-to-liquid crystal phase transition) of the membrane lipids (6-8). Thus, changes in the composition of the fatty acid components of membrane phospholipids could have an adaptive significance in the acclimatization of various types of organisms to temperature (6, 9-12). However, since the modifications of fatty acid composition induced by temperature are generally slight, we have decided to develop a different approach to this problem based on the O₂-triggered modifications of membrane lipids in situ (13-15). Therefore, we determine in this paper the effect of O₂-triggered quantitative transformation of oleate (C₁₈:1) into linoleate (C₁₈:2) in sycamore cells on the transition temperature and Arrhenius activation energy (Eₐ) of 1 the respiration of the intact cells and 2 the succinate dehydrogenase activity of their isolated mitochondria.

MATERIALS AND METHODS

Culture Conditions—Cells from Acer pseudoplatanus L. (sycamore-maple tree) were cultivated at 25 °C in a phytostat for automatic mass culture of plant cells in liquid medium as described previously (16). This apparatus allowed the culture of 20-liter cell suspensions under batch conditions at controlled temperatures. The automatic recording of cell-suspension growth was carried out by means of turbidity measurements (16). The culture medium was prepared according to Lament (17) modified by Lessure (18). In standard cultures, cell suspensions were oxygenated by bubbling air through a sintered glass filter at a rate of 60-80 liters/h. By this way, the O₂ concentration of the standard cultures remained near the saturation (250 μM at 25 °C) during the cell culture growth. For experiments at low O₂ concentrations, special gas mixtures (N₂ + O₂) furnished by Air Liquide (France) were used. The concentration of O₂ dissolved in the culture medium was controlled using a Clark O₂ electrode (Beckman, O₂ analyzer, Fielddab).

Preparation of Mitochondria—Mitochondria were prepared using standard techniques (19). Typical preparations were made from 1 kg of packed sycamore cells. Cells were disrupted for 30 s with a Super-Dispax SD 400 (IKA-Werk, Germany) in a grinding medium consisting of 0.3 mM mannitol, 1 mM EDTA, 20 mM MOPS buffer (pH 7.4), 0.1% defatted bovine serum albumin, and 0.05% cysteine. Mitochondria were purified on sucrose gradients (20). The yield of mitochondria was 20-30 mg of mitochondrial protein/preparation.

O₂ Uptake Measurements—O₂ uptake was measured at 25 °C using a Clark-type O₂ electrode system purchased from Hansatech Ltd., Hardwick Industrial Estate, King's Lynn, Norfolk (21). The reaction medium for the mitochondria (medium A) contained 0.3 mM mannitol, 5 mM MgCl₂, 10 mM KCl, 10 mM phosphate buffer, pH 7.2, and 0.1% (w/v) defatted bovine serum albumin. The reaction medium for the cells was their culture medium. The jacketed reaction chamber was maintained within ±0.2 °C of the desired temperature with a Polystat 22 (Bioblock, France) circulating bath. A convenient and rapid means of accurately determining the O₂ content of the reaction medium is by employing small amounts of mitochondria with limiting concentrations of spectrophotometrically standardized NADH. The high affinity of plant mitochondria for NADH permits a stoichiometric titration of O₂ content at all the temperatures tested. The intactness of mitochondrial preparations was determined by cytochrome c oxidase.

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† The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
dation, using an O₂ electrode according to the method of Neuburger et al. (22). Preparations leaving an apparent percentage of intactness less than 90% were discarded since preliminary work showed that their stability was insufficient.

Lipid Analysis—Cell suspension aliquots were partially dried by filtration under vacuum and fixed by adding boiling ethanol. Cell lipids were extracted according to Folch et al. (23). Mitochondrial suspension aliquots were fixed by boiling for 2 min. Mitochondrial lipids were extracted according to Bligh and Dyer (24). Further phospholipid analysis was performed by two-dimensional chromatography on Silica Gel 60 precoated thin-layer chromatography plates (Merck). A solvent system of chloroform/methanol/water (65:25:4, v/v) was used in the first development and chloroform/acetic acid/methanol/acetic acid/water (100:40:20:20:10, v/v) in the second development. Polar lipids were located under UV light (360 nm) after spraying the plates with anilinonaphthalein sulfonate (0.2% in methanol). Individual polar lipids were identified by their reaction with specific spray reagents and by comparing their Rf values with those of reference standards. Fatty acid methyl esters were made by transesterification of polar lipids at 70 °C for 1 h, in a mixture of methanolsulfuric acid/benzene (100:5:5, v/v). Methyl esters were extracted with hexane and chromatographed on an Internats gas chromatograph (IGC 131) equipped with a hydrogen flame ionization detector and an Intersmat integrator (IRC-1B). Separations were carried out at 175 °C using a column packed with 10% diethylene glycol sulfonate on Varaport 30 Chromosorb. Quantitative analyses of fatty acids and their parent lipids were made according to Allen and Good (25).

**TABLE I**

| Total  | PC    | PE    | PI    | PG    | DPG   |
|--------|-------|-------|-------|-------|-------|
| C₁₀₀  | 22 25 15 18 | 20 23 42 44 46 47 5 7 |
| C₁₅₀  | 3 1 2 2 0.8 | 2 6.8 4 3 6 4 1 1 |
| C₁₁₁  | 53 4 6 3 3.5 54 3.4 30 5 26 5 25 5 |
| C₁₁₂  | 15 45 12 50 | 15 51 | 18 36 17 38 44 62 |
| C₁₁₂* | 7 23 7 27 | 8 21 16 14 5 24 25 |

**TABLE II**

| Total  | PC    | PE    | PI    | PG    | DPG   |
|--------|-------|-------|-------|-------|-------|
| C₁₀₀  | 20 26 18 24 | 23 28 5 7 43 51 46 61 |
| C₁₅₀  | 1.8 1.8 2 1.7 2 1.6 1 1 4.5 2 8 18 4 5 |
| C₁₁₁  | 42 3 54 3 3.8 3 25 5 24 3 26 5 |
| C₁₁₂  | 24 50 16 47 | 27 52 44 62 20 37 17 24 |
| C₁₁₂* | 11 19 9 23 | 10 14 24 25 8.5 8 6 6 |

**FIG. 1.** Effect of O₂ on the transformation of oleate into linoleate and linolenate in sycamore cell. Sycamore cells cultivated on O₂-limiting medium (10 μM O₂) were used (see text). At time zero (t = 0), the culture was submitted to a normal air bubbling (250 μM O₂). Fatty acids were then measured as described under “Materials and Methods.” For each time period, the total amount of polar lipid fatty acids present in sycamore cell fractions remained constant and was 30 μg/10⁶ cells.

**RESULTS**

**Effects of Dissolved O₂ Concentration on Phospholipid and Fatty Acid Composition**—It was first observed that the relative proportion of each phospholipid in sycamore cell and their mitochondria did not vary significantly with the O₂ concentration (from 10 up to 250 μM) of the culture medium (Table I). In contrast, the O₂ concentration of the nutrient medium had a strong effect on the fatty acid composition of membrane lipids. At low O₂ concentrations, the difference in unsaturation was evident with all lipid classes of the cell membranes; the molar proportion of oleate increased dramatically whereas that of linoleate decreased. For example, at 250 μM O₂ (standard culture conditions), oleate and linoleate accounted, respectively, for 4 and 45% (average value in sycamore cells), whereas at 10 μM O₂ (O₂-deficient cultures) they accounted for 53 and 15%. The same was observed with the mitochondria prepared from the two types of cells (C₁₁₂ and C₁₁₂* cells); at 10 μM O₂ concentration in culture medium, the percentage of oleate increased from 3 to 45% and that of linoleate decreased from 49 to 22% (Table II). Even the cardiolipin (diphosphatidylglycerol) which is specific for mitochondria (28) and particularly rich in unsaturated fatty acids (linoleate and linolenate) showed a strong decrease of unsaturation level as a response to low O₂ concentration.

When a normal culture oxygenation was restored (250 μM O₂ instead of 10 μM O₂) oleate underwent a rapid desaturation process (Fig. 1). Under these conditions, the loss of oleic acid was associated with a stoichiometric appearance of linoleate and linolenate. This desaturation was similarly observed with the different polar lipids of the membrane systems. The
maximal rate for the conversion of oleate to linoleate observed at 25 °C was about 4 nmol/h/10^6 cells. Consequently, using Clel cells and either standard (Clel) or Clel, cells submitted to a 24-h O_2-triggered desaturation process (see Fig. 1), it was possible to analyze the effects of decreases in the fraction of unsaturated fatty acids residues in the cells, which are very much greater than the differences reported to exist between chilling-sensitive and chilling-resistant species, on the rate of respiration.

**Respiration Measurements**—Typical sycamore cell respiration rates are shown in Fig. 2. Cells harvested during the exponential phase of growth exhibited high O_2 uptake rates (15-16 nmol of O_2/min/10^6 cells). A respiratory response to carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was observed repeatedly at concentrations of 2 μM; an O_2 uptake stimulation of 100-120% was usually observed. The O_2 uptake rates were about the same for normal and O_2-deficient cells, even after adding an uncoupler (2 μM FCCP) in the medium, although the unsaturated fatty acid residues were considerably reduced in O_2-deficient cells. Furthermore, the final and strong inhibition obtained with cyanide was about the same for both types of cells.

Fig. 3 illustrates several O_2-electrode traces obtained with mitochondria prepared from normal and O_2-deficient cells. We observed that, on a mitochondrial protein basis, the rates of O_2 uptake in state 3 are about the same for normal and O_2-deficient mitochondria. The comparison of the ADP/O ratios and respiratory controls of the two types of mitochondria leads to the same conclusion. In both cases, malate, pyruvate, α-ketoglutarate and NADH were readily oxidized with good respiratory controls and ADP/O ratios comparable to those observed with mitochondria from other plant tissues (28).

**Arrhenius Discontinuities Measurements**—Arrhenius plots of the uncoupled respiration rates of Clel and Clel, cells showed similar patterns (Fig. 4). In both cases, as temperature increased, there was an upward inflection and associated decrease in slope at 18-20 °C (transition temperature). The energies of activation calculated from the two distinct slopes...
exhibited a low affinity for molecular oxygen. The implication of this result is that the phospholipid suggesting a considerable degree of flexibility in the membranes during cell growth without concomitant synthesis of oleoyl to a linolenyl product and that the desaturase(s) involved in the mechanisms of desaturation are 62 and 15 kJ/mol, respectively. Interestingly, the Arrhenius plots of the uncoupled respiration rates of both cell types yielded very similar values for activation energies. Likewise, semi-log plots of the steady-state velocity of succinate oxidation at state III in mitochondria isolated from C_{18-1} and C_{18-2} cells versus (temperature)−1 showed almost identical patterns (Fig. 5). In both cases, there was one break or discontinuity in the Arrhenius plots observed at 26-28 °C and the Arrhenius activation energies were 60 and 14 kJ/mol below and over transition temperatures, respectively.

**DISCUSSION**

These results demonstrate that the aerobic conditions during growth of sycamore cell cultures affected the fatty acid pattern of each phospholipid; by maintaining the oxygen at low concentration (around 10 μM), the molar proportion of oleate (temperature of fusion, 13.4 °C) increased dramatically whereas that of the linolate (temperature of fusion, −5 °C) decreased. Interestingly Harris and James (15) had earlier suggested that the increase in unsaturation at lower temperature could be attributed to an increase in dissolved O\(_2\). Under these conditions, the aeration of the culture medium (250 μM O\(_2\)) induced a rapid transformation of oleate to linoleate associated with all the phospholipids. One is forced to conclude, therefore, that β-oleoyl phosphoglycerides present in cell membrane systems are the most likely candidates for the conversion of oleoyl to a linolenyl product and that the desaturase(s) involved in the mechanisms of desaturation exhibited a low affinity for molecular oxygen. The implications of these findings are that proteins may be inserted into the membranes during cell growth without concomitant synthesis and insertion of normal molecular species of a given phospholipid suggesting a considerable degree of flexibility in the mechanism of membrane growth. However, the question concerning the specific mechanisms involved in the biosynthesis of linoleic and α-linolenic acids remains unanswered, and further work has to be done in order to resolve this problem.

According to several authors (29-31), mitochondrial membranes exhibit a melting transition over a particular temperature range which depends critically on the nature of the fatty acid residues of their polar lipids. Above the upper temperature limit of the thermotropic transition, all the lipids in the membrane bilayer are in a fluid (or random) state. As the temperature is decreased, certain lipids form "solid" patches as their disorder-order phase transition temperatures are reached. Consequently, a very large number of reports have associated thermal transitions in the membrane lipids (determined by spin labeling) with changes in the activities of membrane-bound enzymes. In addition, it has been generally assumed that exposure of plants to lower temperatures leads to an increase in unsaturated fatty acids and that this was a part of the mechanism for avoiding freezing injury. However, the results presented in this paper shed some doubt on a simple relationship between fatty acid composition and membrane fluidity properties. In the case of sycamore cells in suspension cultures, dramatic changes in the degree of unsaturation of the fatty acids in mitochondrial membranes induced by O\(_2\) concentration (which are much more significant than the modifications induced by temperature changes, see Ref. 13) could occur without any change in the Arrhenius-type plots of the rate of mitochondrial respiration (Fig. 5).

In fact, as suggested by different authors (32-36), some of the Arrhenius discontinuities observed at the level of membrane enzymes could be the consequence of intrinsic thermotropic changes in protein arrangement independent of lipid fluidity.
Temperature and Fatty Acid Control of Respiration

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