LIPID PHASE TRANSITION AND BREAKS IN THE ARRHENIUS PLOTS OF MEMBRANE-BOUND ENZYMES IN MITOCHONDRIA FROM NORMAL RAT LIVER AND HEPATOMA AH-130

F. FEO, R. A. CANUTO and R. GARCEA
Istituto di Patologia Generale dell'Università di Torino, Corso Raffaelo 30, 10125 Torino

and

A. AVOGADRO and M. VILLA
Istituto di Fisica Generale dell'Università di Pavia, Pavia

and

M. CELASCO
Istituto di Fisica Generale dell'Università di Torino, Italy

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1. Introduction

The relationships between the physical state of membrane lipids and enzymatic or transport activities have been analyzed in several publications [1–5] using various microorganisms. Discontinuities in the activation energies of enzymatic activities or transport processes were found to depend on the fatty acid composition and cholesterol content of membranes. Such discontinuities sometimes coincided with the phase transition of lipids as detected by X-ray diffraction or thermal analysis [3,5]. However, sometimes disparities between the activity transition temperatures and lipid transition temperatures were observed [1]. Analogous discrepancies have been seen in studies with isolated mitochondria. Different activities of membrane-bound enzymes of mitochondria exhibit breaks in their Arrhenius plots at 23–24°C and at 10–12°C in homeothermic animals and chilling-sensitive plants, respectively [6]. Electron spin resonance experiments showed that the temperatures of the breaks in the Arrhenius plots of some enzymatic activities in rat liver mitochondria coincide with those of the breaks in the Arrhenius plots of spin-label mobility [7]. However, Lenaz et al. [8] found that the breaks in the Arrhenius plots for enzymatic activities of beef heart mitochondria fall in a wide range of temperatures (17.7–27.0°C). Furthermore, Blazyk and Steim [9] observed by differential scanning calorimetry (DSC) that the lipids extracted from rat liver mitochondria show a broad transition between approx. −40°C and +30°C. A transition between approx. −20°C and +30°C was also found in whole organelles. In contrast, with mitochondria from aerobic yeast no transition was detected by DSC [10], while X-ray diffraction experiments indicated a transition from −15–−5°C [11].

The experiments reported here were done to evaluate whether, (a) membrane-bound enzymes of rat liver mitochondria behave differently with respect to the temperature of the sudden change in the activation energy, (b) there exists a correlation between the breaks in the Arrhenius plots and any unique portion of the calorimetric peak of the lipid phase transition, or (c) cholesterol-rich membranes,

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such as those of hepatoma mitochondria [12,13], show some modifications of both lipid and enzymatic activity transitions.

2. Materials and methods

Long-Evans rats (150–220 g) were used as source of normal liver and to receive Yoshida hepatoma AH-130 transplants. The medium and procedures for isolation of mitochondria and mitochondrial membranes (inner plus outer) were as published [12]. The concentrations of microsomes, from determinations of glucose-6-phosphatase and NADPH-cyt. *c* reductase, was 4.5–5.6% in both liver and hepatoma mitochondria.

For differential thermal analysis (DTA), mitochondria were suspended in the isolation medium (220 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, pH 7.4, 2 mM EDTA and 0.1% defatted bovine serum albumin) containing either 50% ethylene glycol or 40% glycerol and centrifuged at 115 000 × *g* for 50 min. Dehydrated mitochondrial membranes were obtained by storing pellets of wet membranes overnight under vacuum over P_2O_5. The final water content of dehydrated membranes was (wt%) 5–10%. Samples of 55–86 mg (90–140 μg of lipid phosphorus for whole organelles and 200–350 μg for dried membranes) were sealed in silver calorimeter pans.

The preparation of lipid-deficient mitochondria (LDM) by treatment with phospholipase A_2 (Koch-Light) and the binding of egg phosphatidyl choline (EPC) to the defatted membranes were carried out as described by Fleischer and Fleischer [14]. EPC was extracted and purified according to Singleton et al. [15]. Samples for DTA were prepared by sedimenting EPC-bound mitochondria, suspended in sucrose-glycol 1:1 (v/v), at 165 000 × *g* for 2 h. Lipids were extracted under nitrogen as previous [16]. 20–40 mg of mitochondrial lipids or 25 mg of EPC dissolved in chloroform, were evaporated under nitrogen in a test tube and the least traces of solvent removed in vacuo. 50–100 μl of water–ethylene glycol 1:1 (v/v) were added to the dry films. The lipids were dispersed by vortexing the stoppered tubes for 15 min above the phase transition temperature of the lipids. Aliquots of 7–30 mg (wet weight) of the dispersions (49–200 μg of lipid phosphorus) were sealed in silver calorimeter pans.

DTA runs were performed with a Du Pont 900 apparatus. A scan speed of 10°C/min was used with a ΔT sensitivity of 0.1°C/min. A number of ascending and descending runs were performed on each sample, always obtaining the same results. The data presented in figures refer only to ascending runs.

Succinate-cyt. *c* reductase and NADH-cyt. *c* reductase (rotenone-insensitive) were determined according to Sottocasa et al. [17]. The reaction was followed in a Beckman Acta CIII spectrophotometer equipped with a thermostatic cell. Mitochondria (0.1–0.5 mg protein) submitted to either a cycle of freezing and thawing or to brief sonication, were used to determine the ATPase in 1 ml of a reaction mixture containing 50 mM Tris–acetate, pH 7.4, and 5 mM $MgSO_4$. The reaction was initiated by addition of ATP up to a concentration of 5 mM and was arrested, after 5 min incubation in a water bath, by 5 ml of 6% trichloroacetic acid. Temperatures in the assay media were controlled by a thermocouple. Inorganic phosphorus was determined according to Beremblum and Chain [18], phospholipid as described elsewhere [16] and protein by a biuret method [19].

3. Results

As shown in fig.1, breaks in the Arrhenius plots for succinate-cyt. *c* reductase, NADH-cyt. *c* reductase

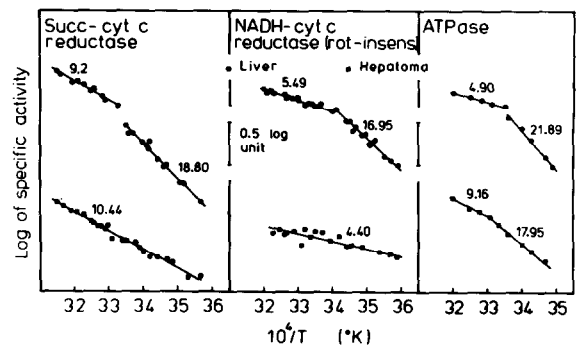

Fig.1. Arrhenius plots of succinate-cyt. *c* reductase, NADH-cyt. *c* reductase (rotenone-insens.) and Mg^{2+}-dependent ATPase of rat liver and hepatoma mitochondria. Succinate and NADH-cyt. *c* reductase activities were measured as nmol of cyt. *c* reduced/min.mg of protein; ATPase was measured as nmol of P_i hydrolyzed/min.mg of protein. Numbers over the lines indicate the activation energies. Each point represents the mean value of three to eight experiments.

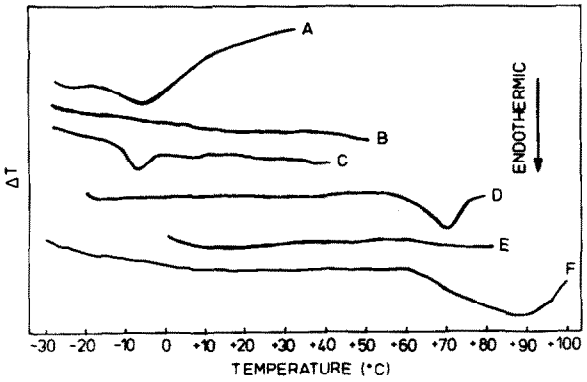

Fig.2. DTA heating curves for extracted lipids, whole mitochondria and dehydrated mitochondrial membranes. Lipids were dispersed in water–glycol, 1:1, mitochondria and mitochondrial membranes were suspended in sucrose buffer–glycol, 1:1; (A) Total lipids from liver mitochondria, 140 μg of lipid P. (B) Total lipids from hepatoma mitochondria, 200 μg of lipid P. (C) Phospholipids from hepatoma mitochondria, 176 μg of lipid P. (D,E) Whole liver mitochondria, before (D) and after (E) heat denaturation of protein, 122 μg of lipid P. (F) Dehydrated membranes from liver mitochondria, 232 μg of lipid P, approximate final unfreezable water content of membranes dried in vacuo (wt%) 8%.

(rotenone-insens.) and ATPase of liver mitochondria occur at 26–28°C, 20°C and 24°C, respectively. In hepatoma mitochondria no discontinuities in the Arrhenius plots were found for succinate- and NADH-cyt. *c* reductases, while a break at 29°C was observed for ATPase. However, the difference between the activation energies for this enzyme below and above the transition temperature is about two times lower than in liver mitochondria.

The DTA spectrum for total lipids extracted from liver mitochondria is shown in fig.2A. There is a transition starting at approx. −17°C and centered around −6°C. In total lipids from hepatoma mitochondria no transitions were observed (fig.2B), even though larger samples were analyzed than with lipids from liver mitochondria. However, with the phospholipid fraction of hepatoma mitochondria a transition beginning at about −12°C and centered at approx. −8°C is visible (fig.2C). With whole liver mitochondria a transition starts at approx. +55°C and is centered around +70°C (fig.2D). No transitions take place at lower temperatures. Protein denaturation seems to be involved, since this transition vanishes after heating at 80°C (fig.2E). Several DTA scans were repeated at faster scanning rates (15°C/min), in order to increase the sensitivity and/or with larger samples of mitochondria (140 μg of lipid phosphorus). Some experiments were also performed with mitochondria suspended in 40% glycerol (range of scanned temperatures: −30–+30°C). In all instances the results were identical to those in fig.2D.

To investigate the possibility that ethylene glycol or glycerol influence the thermal behavior of our mitochondrial preparations, some experiments with dehydrated liver mitochondrial membranes were done. Dehydrated membranes contain a small amount of bound water that does not freeze at 0°C. Moreover, samples with high lipid phosphorus content (up to 350 μg) can be prepared, since the loss of water results in an increase of phospholipid per unit of weight. Dried membranes show a broad transition at about +60°C, centered around +90°C (fig.2F) which could be related to extensive chemical degradation as with the +55°C transition in whole mitochondria. The absence of transitions below +55°C was also observed in whole mitochondria or dried mitochondrial membranes from hepatoma (not shown).

Therefore, it seems that lipids which undergo a thermotropic transition apparently change this behavior when included in mitochondrial membranes. This was also observed by experiments with LDM. Treatment with phospholipase A_2 induces a loss of 92.3% of phosphatidyl choline, 88.0% of phosphatidyl ethanolamine and 64.1% of diphosphatidyl glycerol. As a consequence, an 84.7% decrease of total phospholipid occurs. On binding of EPC, mitochondrial phospholipid content increases to 0.103 (S.D. = ± 0.031)

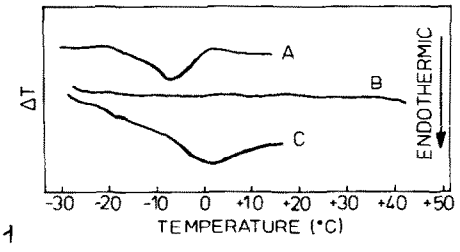

Fig.3. DTA heating curves for egg phosphatidyl choline (A), egg phosphatidyl choline bound to LDM (B) or mechanically mixed with a pellet of LDM (C). (A) 104 μg of lipid P, (B) 86 μg of lipid P, 15 mg of protein, (C) 49 μg of lipid P, 15 mg of protein.

mg/mg of protein versus 0.170 (S.D. = ± 0.018) mg/mg of protein in intact mitochondria. As illustrated in fig.3A, a transition beginning at about −20°C and centered around −6°C takes place in EPC. The binding of this phospholipid to delipidated mitochondrial membranes results in the disappearance of the phase transition (fig.2B). Instead, EPC vesicles, mechanically mixed with a pellet of LDM give the usual transition, although in a higher range of temperatures (fig.3C).

4. Discussion

A discontinuity in the activation energies of succinate oxidase, cytochrome *c* oxidase and ATPase of rat liver mitochondria has been detected at 23–24°C [6]. Our results confirm a break at 24°C for ATPase. Succinate-cyt. *c* reductase and NADH-cyt. *c* reductase (rotenone-insens.) gave breaks at 26–28°C and 20°C, respectively. Thus, in liver mitochondria as in those from beef heart [8], the activity transition of some enzymes may occur at different temperatures. In hepatoma mitochondria the discontinuities of activation energies are slight or absent. This could be due to the high cholesterol content of these organelles [12,13]. Membranes of hepatoma AH-130 mitochondria contain 25 mol percent cholesterol to phospholipid (18.5 inner membrane, 41.7 outer membrane) versus 4.6 mol percent (3.8 inner, 10.9 outer) in liver mitochondria [13]. Rottem et al. [3] failed to detect discontinuities in the activation energy of ATPase in a native strain of *Mycoplasma mycoides* containing 20–25 mol percent cholesterol to total membrane lipid. Transitions were detected in membranes of an adapted strain containing only 3 mol. percent cholesterol. Enthalpy of the phase transition decreases or vanishes in both artificial [20,21] and natural membranes [3,22] with high cholesterol contents. In agreement with these findings, no transitions in total lipids from hepatoma mitochondria were detected, while the DTA curves for phospholipids extracts, free of cholesterol, showed a transition.

The formation of different lipid clusters in some functional areas of the mitochondrial membranes has been proposed to explain the differences in the activity transitions of enzyme [1,8]. Alternatively, enzymes might not all be affected to the same extent and in the same way by the physical state of the bulk of lipids. However, the latter interpretation contrasts with the observation that the lipids bound to yeast-mitochondrial ATPase protein are those that regulate the activity of the intact organelle [11]. The involvement of the bulk of the lipids in the regulation of enzymatic activities also seems to be discounted by the results of the thermal analysis of liver mitochondria. Apparently, there is no transition in whole mitochondria and in dehydrated membranes below 55°C. A transition was observed in extracted lipids at temperatures far from those of the breaks in the Arrhenius plots of enzymatic activities.

The absence of a thermotryptic of lipid in whole mitochondria was also described by Bertoli et al. [10]. Possible attributable to an effect on the energy of transition of largely hydrophobic lipid–protein interactions. This is supported by the observation that the binding of egg phosphatidyl choline to LDM causes the 'melting' of phosphatidyl choline to disappear. It has been shown [11,23] that the molecular motion of acyl chains adjacent to the lipid–protein interface is greatly inhibited. A decrease in the transition enthalpy of phospholipids on interaction with several proteins has also been demonstrated [24,25]. Among such proteins there is cytochrome *c* as well as proteins which undergo extensive hydrophobic contacts with phospholipids, as do a large fraction of mitochondrial proteins [26]. Proteins were shown to represent, on a weight basis more than 80% of mitochondrial membrane constituents [27]. One can imagine that, on interaction with proteins, the number of phospholipid molecules participating in the cooperative melting of the bulk of lipids is greatly reduced, so that no thermal transition will be detected.

Our calorimetric data do not agree with those of Blazyk and Steim [9] who detected transitions between −20°C and +30°C in whole-liver mitochondria using DSC. The absence of thermal transitions below +55°C in our preparations of whole mitochondria or dried membranes does not appear to be an instrument artifact. In fact, transitions were detected in samples of extracted lipids which had the same or even lower lipid phosphorus contents than those of whole mitochondria or dried membranes. Blazyk and Steim [9] found that the membrane transition is 80% as energetic as that of extracted lipids. However, it

seems that this figure should be significantly lower for our mitochondrial preparations. That this difference depends on factors which are known to affect the physical state of lipids and lipid–protein complexes could be suggested. For instance, the protein concentration in membranes [24], pH or the content of divalent cations [28,29], the functional conditions [30] and differences in the lipid and protein composition of the mitochondrial preparations [31]. There is however, no proof that any of these suppositions are correct.

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