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ROLE OF PROTEINS AND LIPIDS IN NON-LINEAR ARRHENIUS PLOTS OF *DROSOPHILA* MITOCHONDRIAL SUCCINATE-CYTOCHROME *c* REDUCTASE STUDIED BY REBINDING EXPERIMENTS

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Summary

Abrupt changes in the Arrhenius activation energy of membrane-bound enzymes have often been correlated with changes in the physical state of membrane phospholipids. Similar changes in activation energy have also been found in soluble enzymes. The possibility exists, therefore, that in some of the membrane-bound enzymes the changes might reflect intrinsic changes of the proteins independent of changes in the membrane phospholipids. This hypothesis was investigated using *Drosophila* mitochondria isolated from wild type and the mutant $Ocd^{ts\text{-}1}$. In this mutant it has been shown that succinate-cytochrome *c* reductase exhibits a change in Arrhenius activation energy at 18°C which is not found in the wild type (Sondergaard, L., Nielsen, N.C. and Smillie, R.M. (1975) FEBS lett. 50, 126—129). A quantitative thin-layer chromatographic analysis of mitochondrial phospholipids showed sphingomyelin to be more abundant in the wild type than in the mutant (5.2% and 4.3% of the total phospholipids, respectively). Since it was shown that the succinate-cytochrome *c* reductase had a lipid requirement for full activity, reciprocal rebinding experiments were done. These experiments showed that the reconstituted membranes exhibited the change in activation energy at 18°C only when the protein moiety came from mutant mitochondria, that is, the change was independent of the source of the phospholipids used.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

Abrupt changes in the Arrhenius activation energy of membrane-bound enzymes have been found for many enzymes in a wide array of organisms (for reviews see e.g. Refs. 1 and 2) and have often been correlated with changes in viscosity of membrane phospholipids (see e.g. Ref. 3). The temperature at which viscosity changes take place has been correlated with the relative proportions of the different lipid classes [4—11] as well as the length and saturation of the fatty acyl side chains of the phospholipids [6,12—18]. Temperature studies with soluble enzymes, on the other hand, have revealed a number which also exhibits temperature-induced changes in Arrhenius activation energy [19—25]. One might expect, therefore, that the changes in Arrhenius activation energy of enzymes in biological membranes might in some cases be due to changes in the enzyme conformation independent of phase changes in the phospholipid moiety of the membrane.

Recently a mutant of *Drosophila melanogaster*, *Out-cold*$^{ts\text{-}1}$ (*Ocd*$^{ts\text{-}1}$), was found [26] which exhibits a change in the Arrhenius activation energy of mitochondrial succinate-cytochrome *c* reductase activity at a temperature much higher than found in the wild type [27]. In light of the above discussion this change could be due to differences between mutant and wild-type mitochondria in phospholipid composition with respect to the polar head groups, length and/or saturation of the fatty acyl side chains or in the temperature sensitivity of the enzymes involved in the succinate-cytochrome *c* reductase activity. The mutant appeared an ideal tool to elucidate whether changes in Arrhenius activation energy are due to a phase transition in the lipid moiety of the membrane or to an inherent change in the proteins. This requires removal of the phospholipids from the intact membranes of mutant and wild-type mitochondria, followed by rebinding membrane lipids from either source to both wild-type and mutant lipid-deficient membranes [28] and then defining any consequent changes in the Arrhenius plots. This paper describes the results of such experiments and also includes a detailed TLC analysis of the phospholipid composition of *Drosophila* mitochondria.

Materials and Methods

The flies used in the present investigation were isolated from highly inbred stocks of *D. melanogaster* either homozygous for the balancer chromosome *Basc* [29] or carrying this chromosome and an X-chromosome with the dominant *Ocd*$^{ts\text{-}1}$ mutation. The flies were 5—10 days of age, a period of the adult *Drosophila* life span where only minor age-dependent changes have been observed in the structure and function of the mitochondria [30,31]. Mitochondria were isolated from 2—5 g adult *Drosophila* flies as described previously [27] with minor modifications. The homogenized flies were centrifuged three times at 500 × *g* and mitochondria were then obtained by sedimentation at 10 000 × *g*. The mitochondrial pellet was washed twice and finally resuspended in a small volume of isolation buffer (0.25 M sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.5 [27]). The purity of the pellet was checked by electron-

microscopy which showed less than 10% contamination (by vol.) with other membranes.

Phospholipid extraction. Mitochondria were extracted with chloroform/methanol (2 : 1, v/v) in the presence of 0.01% butylated hydroxytoluene as an antioxidant as described by Rouser and Fleischer [32].

All operations with phospholipids were performed at room temperature and as much as possible in an atmosphere of N_2. All glassware was washed in chloroform/methanol (2 : 1, v/v) before use and chloroform redistilled into 0.1% methanol before use.

Thin-layer chromatography (TLC). Analyses of membrane lipids were done by two-dimensional TLC on activated plates spread with Silica gel H (Merck) containing 2.5% $Mg_2O_8Si_3$ [33]. Each plate was spotted with a 100—200 µl aliquot of a concentrated lipid extract. Recovery was determined as described previously [34]. The plates were developed in the first dimension with chloroform/methanol/28% aqueous ammonia (65 : 35 : 9, by vol.) and in the second dimension with chloroform/acetone/methanol/acetic acid/water (10 : 4 : 2 : 2 : 1, by vol.). Spots were visualized by spraying with 50% H_2SO_4 containing 0.2% $K_2Cr_2O_7$ and charred for 20 min at 180° C [33].

The different phospholipid classes were identified by comparison with standards (obtained from Sigma (London) Chemical Co. Ltd.) and by spraying with ninhydrin [35] and Dragendorf [36] reagents.

Quantitative determinations were made by phosphorous analysis of the detected lipid spots as described by Rouser et al. [34]. To avoid non-lipid phosphorous contamination Merck HCl Suprapur and Merck $HClO_4$ Suprapur were used and all glassware was acid washed.

Phospholipid dispersion. The mitochondrial lipid extract was fractionated on a Unisil column as described [33]. The chloroform fraction was shown by TLC to lack any phospholipids and was therefore discarded. The acetone and methanol eluates were combined and dried under reduced pressure. Microdispersions of the extracted lipids were formed by adding 5 ml of Tricine/EDTA buffer (0.02 M Tricine, 10 mM EDTA, pH 8.0) to the dried lipids and then sonicate for 20 min in a sonicating waterbath (Branson model H50-S, 50 W at 40 kHz). The temperature in the waterbath was kept between 25 and 30° C. Finally, the microdispersion was clarified by centrifugation for 30 min at 40 000 × *g* and stored under N_2 at 4° C until use.

Removal and rebinding of mitochondrial phospholipids. The phospholipid dependence of mitochondrial succinate-cytochrome *c* reductase was investigated by incubating mitochondria with phospholipase A_2 prepared from *Naja naja* venom (Sigma (London) Chemical Co. Ltd.) as described except that bovine serum albumin was omitted [28]. After different lengths of time aliquots were withdrawn and the membranes sedimented at 20 000 × *g*. The pellet was washed four times with bovine serum albumin buffer (1% albumin, 0.25 M sucrose, 50 mM glycylglycine, 1 mM EDTA, pH 7.4) [28] followed by one wash in isolation buffer [27].

To render the mitochondria lipid deficient, they were extracted with acetone containing 10% water [28] (2 ml/mg protein) for 5 min at 0° C. The membranes were then sedimented by a brief centrifugation at 10 000 × *g* and resuspended in isolation buffer [27]. After two washes the mitochondria were resus-

pended in isolation buffer at a concentration of approx. 1 mg protein/ml. In preliminary experiments the acetone extract was shown by TLC to contain all lipid classes present in the intact membranes indicating that all phospholipids were extractable with this method.

Phospholipids were rebound to the acetone-treated mitochondrial membranes by incubating the phospholipid microdispersions with the lipid-deficient membranes (100—200 μg phospholipid phosphorous/mg protein) at 32°C for 15 min as described elsewhere [28]. Finally non-bound phospholipids were removed by sedimenting the membranes at 20 000 × *g* for 30 min and the membranes resuspended in a small volume of isolation buffer [27].

Miscellaneous methods. Succinate-cytochrome *c* reductase was assayed spectrophotometrically from 10 to 30°C as described previously [27]. In some experiments coenzyme Q_{10} obtained from Sigma (London) Chemical Co. Ltd. was added to the assay mixture (0.05 ml of 0.02% in methanol/3 ml assay media) [28]. Protein was determined by the use of Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA).

Results and Discussion

Phospholipid composition of Drosophila mitochondria

In the $Ocd^{ts\text{-}1}$ mutant a change in the Arrhenius activation energy of the mitochondrial succinate-cytochrome *c* reductase is found at 18°C [27]. In the temperature interval investigated in this report (10—30°C) no change in activation energy is found in wild-type mitochondria [27]. This discrepancy could be due to differences in phospholipid composition of the two types of mitochondrial membranes [4—11].

A quantitative two-dimensional TLC analysis of the total lipids extracted from mitochondria membranes of heterozygous $Ocd^{ts\text{-}1}$/*Basc Drosophila* flies and control *Basc/Basc* flies showed phosphatidylethanolamine, phosphatidylcholine and diphosphatidylglycerol to account for approximately 50%, 18% and 11% of the total phospholipids, respectively (Table I). These results are in agreement with observations on other species of *Diptera* [37—43].

The only significant difference in phospholipid composition between $Ocd^{ts\text{-}1}$/*Basc* and *Basc/Basc* fly mitochondrial membranes is found in the amount of sphingomyelin (Table I).

There might however also be differences between mutant and wild type in length and saturation of the fatty acyl side chains of the phospholipids. Such differences would not be detected by a TLC analysis but their effect on the shape of the Arrhenius plots could be analysed by doing lipid-depletion rebinding experiments if it could be shown that succinate-cytochrome *c* reductase in *Drosophila* mitochondria had a lipid requirement for full activity [28]. Such experiments would also test the effect of the observed small difference in the sphingomyelin content (Table I).

Phospholipid requirement of succinate-cytochrome c reductase

To investigate the phospholipid dependence of mitochondrial succinate-cytochrome *c* reductase in *Drosophila*, mitochondria were incubated with phospholipase A_2 and aliquots withdrawn after different lengths of time. The results

TABLE I

LIPID COMPOSITION OF *DROSOPHILA* MITOCHONDRIA ISOLATED FROM $Ocd^{Ts-1}/Basc$ AND *Basc/Basc* FLIES

Mitochondria were extracted with chloroform/methanol (2 : 1, v/v) and the lipid extract analysed by two-dimensional TLC. Only TLC plates on which recovery was better than 98% were used. Results are expressed as percent of total phosphorous ± S.D. (n = 20). P is the probability, non-paired t-test comparing $Ocd^{ts-1}/Basc$ and *Basc/Basc*. PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPG, diphosphatidylglycerol; Sph, sphingomyelin; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PA, phosphatidic acid; FFA, free fatty acids; LPL, less polar lipids; NPL, neutral lipids; n.s., not significant.

Lipid class	$Ocd^{ts-1}/Basc$	*Basc/Basc*	P
PE	51.5 ± 1.8	51.5 ± 1.7	n.s.
PC	18.1 ± 0.8	18.3 ± 0.8	n.s.
DPG	10.8 ± 0.9	11.1 ± 0.8	n.s.
Sph	4.3 ± 0.7	5.2 ± 0.9	0.05
LPE	2.2 ± 0.2	2.5 ± 0.3	n.s.
LPC	0.8 ± 0.2	0.9 ± 0.3	n.s.
PG	1.7 ± 0.3	1.7 ± 0.3	n.s.
PI	2.5 ± 0.2	2.6 ± 0.2	n.s.
PS	2.3 ± 0.3	2.4 ± 0.3	n.s.
LPI	0.41 ± 0.10	0.44 ± 0.03	n.s.
LPS	0.42 ± 0.04	0.42 ± 0.11	n.s.
PA	0.22 ± 0.05	0.20 ± 0.05	n.s.
FFA	0.23 ± 0.09	0.27 ± 0.06	n.s.
LPL	0.59 ± 0.09	0.60 ± 0.11	n.s.
NPL	0.31 ± 0.05	0.29 ± 0.04	n.s.
Unknown	1.6 ± 0.2	1.6 ± 0.2	n.s.
Origin	0.63 ± 0.03	0.58 ± 0.08	n.s.

of such an experiment are shown in Fig. 1. As expected from earlier reports [28] the specific activity decreased as the phospholipid content of the membranes decreased indicating a requirement for phospholipids. The enzyme activity dropped to about 30% of the original value when only 50% of the phospholipids had been removed. This decrease in activity was faster than that ob-

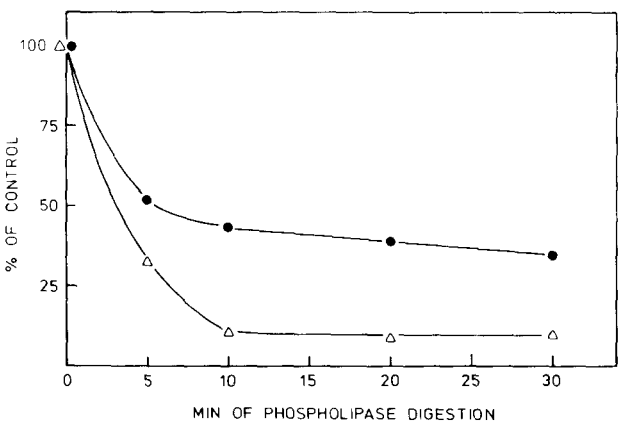

Fig. 1. Specific activity (△) and phospholipid content (●) of *Drosophila* mitochondrial membranes incubated with phospholipase A_2 for different lengths of time as indicated. Results are expressed as percent of a control incubated under similar conditions except no phospholipase A_2 was added.

served for the same enzyme activity in mammalian mitochondria where as much as two thirds of the phospholipids should be removed before an appreciable phospholipid requirement was observed for succinate-cytochrome *c* reductase [28]. The dramatic effect of phospholipase A_2 might be due to the inhibitory action of breakdown products of the phospholipids [28]. In an attempt to more efficiently remove the breakdown products a batch of phospholipase-treated mitochondria was washed twice more in albumin buffer [28] (i.e. six times in all). This procedure, however, did not change the marked lipid dependence. On the other hand, mitochondria extracted with 10% acetone [28] showed essentially the same strong dependence of phospholipids for the succinate-cytochrome *c* reductase activity (data not shown). To completely avoid the possibility of an inhibitory effect of the lysophosphatides or free fatty acids, the rebinding experiments were done using the acetone extraction procedure [28].

Rebinding experiments

The results shown in Table II demonstrate that acetone-extracted membranes can be reactivated by rebinding of phospholipids provided coenzyme Q_{10} is present in the assay mixture. This demonstrates a requirement for both phospholipids and coenzyme Q_{10} of succinate-cytochrome *c* reductase activity of acetone-extracted membranes from insect mitochondria. This finding is in agreement with results from experiments involving a large number of membrane-bound enzymes in other organisms (for reviews see Refs. 1 and 2). The essentially complete recovery of activity upon the addition of coenzyme Q_{10} plus phospholipids indicated that no or very little protein denaturation took place during the acetone treatment.

Temperature studies were done on the succinate-cytochrome *c* reductase activity using reactivated acetone-treated membranes, and Arrhenius plots constructed for the temperature interval 10—30°C. As can be seen in Fig. 2A and B and Table III, $Ocd^{ts\text{-}1}$ membranes reactivated with $Ocd^{ts\text{-}1}$ phospholipids and control membranes reactivated with control phospholipids exhibited the same temperature characteristics as the intact $Ocd^{ts\text{-}1}$ and control mitochondrial membranes, respectively [27], and the Arrhenius activation energies of the

TABLE II

PHOSPHOROUS CONTENT OF CONTROL AND LIPID-DEFICIENT *DROSOPHILA* MITOCHONDRIA AND SPECIFIC ACTIVITIES OF SUCCINATE-CYTOCHROME *c* REDUCTASE UNDER CONDITIONS AS INDICATED

The assay mixture contained: approx. 200 μg mitochondrial protein, 30 mM phosphate buffer (pH 7.5), 0.6 mM Tris, 0.03 mM EDTA and 0.05% KCN. The reaction was initiated by addition of 45 mM sodium succinate. The final volume was 3 ml. PL, phospholipids; CoQ_{10}, coenzyme Q_{10}.

Mitochondrial membranes	μg P/mg protein	Specific activity at 25°C (μmol succinate oxidized/min per mg protein)			
		No	+CoQ_{10}	+PL	+PL+CoQ_{10}
Control	20.3	0.326	0.310	—	—
After acetone extraction	5.3	0.007	0.016	0.014	0.320

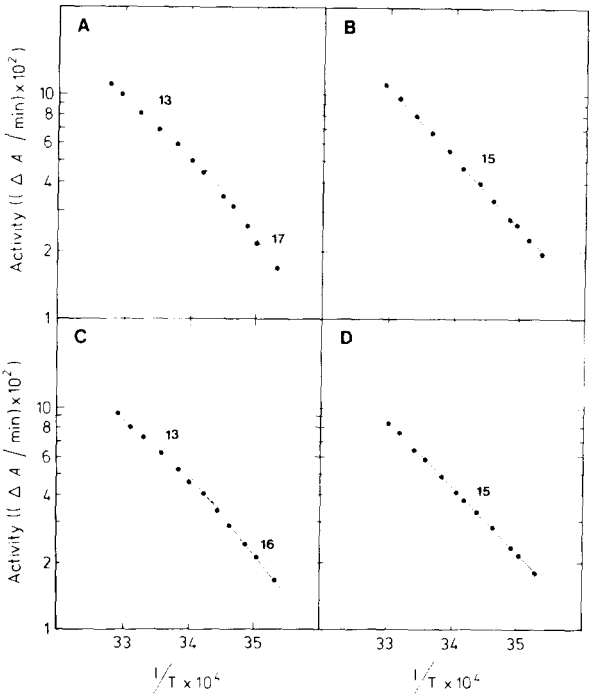

Fig. 2. Arrhenius plots of succinate-cytochrome *c* reductase activity of *Drosophila* mitochondrial membranes extracted with 90% acetone and reactivated with phospholipids. (A) Extracted Ocd^{ts-1} membranes reactivated with Ocd^{ts-1} lipids. (B) Control (*Basc/Basc*) membranes reactivated with control lipids. (C) Ocd^{ts-1} membranes reactivated with control lipids. (D) Control membranes reactivated with Ocd^{ts-1} lipids. The succinate-cytochrome *c* reductase was assayed as described in Table II. The Arrhenius curves were determined by the least-square method [44].

TABLE III

TRANSITION TEMPERATURE AND ARRHENIUS ACTIVATION ENERGIES FOR SUCCINATE-CYTOCHROME *c* REDUCTASE ABOVE AND BELOW TRANSITION FOR INTACT AND REACTIVATED LIPID-DEFICIENT *DROSOPHILA* MITOCHONDRIA ISOLATED FROM Ocd^{ts-1} AND CONTROL FLIES

The lipid-deficient membranes were recombined with phospholipids from both Ocd^{ts-1} and control mitochondria and Arrhenius plots constructed. Assay conditions are in Table II.

Source of mitochondrial membranes	Source of phospholipids	Transition temperature (°C, ± S.D.)	Arrhenius activation energy (kcal/mol, ± S.D.)	
			Lower	Upper
Basc	*Basc*	—	15 ± 1	—
Basc	Ocd^{ts-1}	—	15 ± 1	—
Ocd^{ts-1}	*Basc*	18 ± 1	17 ± 1	13 ± 1
Ocd^{ts-1}	Ocd^{ts-1}	18 ± 1	17 ± 1	13 ± 1
Intact mitochondria *		none	15 ± 1	—
Basc Intact mitochondria * Ocd^{ts-1}		18 ± 1	16 ± 1	13 ± 1

* Data taken from Ref. 27.

reconstituted membranes do not differ significantly from those obtained for the intact membranes (Table III) [27]. It is reasonable to believe that the phospholipids required for the reactivation of lipid-deficient membranes are also the lipids suggested to be responsible for abrupt changes in the Arrhenius activation energy of the enzymes studied [1]. Arrhenius plots of experiments in which phospholipids from mitochondria of control *Drosophila* or the mutant $Ocd^{ts\text{-}1}$ were rebound to lipid-deficient membranes from mutant or control, respectively, do not support this idea. The occurrence of a change in Arrhenius activation energy in these experiments was dependent upon the source of the lipid-deficient membranes. That is, if the membranes came from control mitochondria no change in activation energy was found in the temperature range 10–30°C but if the membranes came from $Ocd^{ts\text{-}1}$ mitochondria a change was obvious at 18°C irrespective of the source of the rebound lipids. In all experiments the membranes were fully activated by the foreign phospholipids.

Thus in *Drosophila* the change found in the Arrhenius activation energy at 18°C of succinate-cytochrome *c* reductase in mutant mitochondria is due to a change in the membrane proteins independent of changes in the membrane lipids. Yamaki and Uritani [45] working with sweet potato mitochondria have made similar observations. That is, a change at about 17°C in succinate oxidation was independent of the source of the phospholipids in rebinding experiments. In accordance with these experiments acclimatization studies on gold fish have shown that while a break in the Arrhenius plot of mitochondrial respiration changes with acclimatization temperature, the fluidity of the membranes does not change accordingly, as judged by the saturation index of membrane phospholipids [46]. Similarly it has been shown that a break found in the Arrhenius plots of Ca^{2+}-ATPase of sarcoplasmic reticulum occurred at the same temperature even after more than 95% of the membrane phospholipids had been replaced with detergent [47] or when the membranes had been reactivated with foreign phospholipids [48]. Working with 5'-nucleotidase Stanley and Luzio showed that the temperature of a change in Arrhenius activation energy was identical in a detergent-solubilized preparation, a sphingomyelin complex and the intact membrane-bound enzyme [49]. These experiments, then, together with the present report indicate that at least for some membrane-bound enzymes abrupt changes in Arrhenius activation energy are independent of changes which could take place in associated phospholipids.

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