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THE EFFECT OF PARTIAL DEGRADATION OF MITOCHONDRIAL PHOSPHOLIPIDS BY PHOSPHOLIPASE A ON THE TEMPERATURE DEPENDENCE OF SUCCINATE–CYTOCHROME *c* REDUCTASE AND CYTOCHROME *c* OXIDASE

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SUMMARY

Rat-liver mitochondria, devoid of outer membrane, were subjected to the action of pure phospholipase A_2 from porcine pancreas. Degradation products were removed by treatment with bovine serum albumin.

Arrhenius plots of succinate–cytochrome *c* reductase and cytochrome *c* oxidase activities revealed two breaks, in the temperature region 10–30 °C. The exact values of the break temperatures were dependent on the extent of phospholipid hydrolysis, particularly in the case of succinate–cytochrome *c* reductase.

The higher temperature break probably reflects a phase change of the lipids in close proximity to the enzyme molecules, while the lower temperature break can possibly be explained by a reorganization of the lipids in the gel phase.

The values of the apparent activation energies varied with the extent of phospholipid hydrolysis and, consequently, with the relative phospholipid composition, indicating that the magnitude of the activation energy of a lipid-dependent enzyme is not merely governed by the physical state, but also by the composition of the lipid environment.

Differences were observed in the effect of phospholipase treatment between the two enzyme activities studied. This suggests a certain degree of heterogeneity in the distribution of the lipids which are directly involved in the functioning of the enzyme molecules.

INTRODUCTION

Recently accumulated evidence indicates that the properties of membrane-bound functional proteins can be influenced by the physical state of the membrane lipids [1–5]. Studies on essential fatty acid auxotrophs of *Escherichia coli* revealed that temperatures characterizing breaks in Arrhenius plots of transport activities are determined by the fatty acid supplement on which the cells are grown [1, 2, 5]. A correlation between the temperature characteristics of physiological properties of

E coli and the physical nature of isolated membrane lipids was directly demonstrated by monolayer studies of Overath et al [1] Breaks in the slopes of Arrhenius plots were interpreted as reflecting phase transitions of the membrane lipids [1–5] There are indications that the temperatures at which the slopes of Arrhenius plots abruptly change depend on the average composition of the membrane lipids; the temperature dependence of transport processes is likely to be responsive to the physical state of the bulk of the lipid bilayer, randomized by lateral diffusion of the phospholipids and therefore homogeneous in composition [4] Similar to transport phenomena membrane-associated enzyme activities can display discontinuous changes in the slopes of their Arrhenius plots The characteristic break temperatures, however, of for instance various mitochondrial enzymes are not identical according to investigations of Lenaz et al [6] and Watson et al [7], suggesting a heterogeneous lipid distribution In contrast, a study of Raison et al [8] suggests that there is no variation in the break temperatures of a number of enzymes in rat-liver mitochondria

To obtain more information about the influence of lipids on membrane-bound enzymes we studied the effect of partial degradation of the inner-membrane phospholipids by phospholipase A_2 on the temperature dependence of succinate–cytochrome *c* reductase and cytochrome *c* oxidase activities of rat-liver mitochondria

MATERIALS AND METHODS

Preparation of inner-membrane-matrix particles

Male Wistar rats (200–300 g) were injected intraperitoneally with 10 μCi [1,2-$^{14}C_2$]ethanolamine (30 Ci/mole, Radiochemical Centre, Amersham, England) in 0 5 ml 0 15 M NaCl After about 16 h the animals were killed by decapitation, livers were homogenized in 0 25 M sucrose, 5 mM Tris, 1 mM EDTA, pH 7 4 After removal of nuclei and cell debris by centrifugation for 10 min at 850 $\times g$, mitochondria were pelleted at 4500 $\times g$ during 10 min and washed twice Outer membranes were removed by digitonin treatment according to Schnaitman and Greenawalt [9] Monoamine oxidase determinations [10] revealed that usually 90–95 % of the outer membrane material was removed by this treatment Protein was determined by the biuret method [11]

Treatment with phospholipase A

Mitochondria stripped of outer membrane were subjected to the action of phospholipase A The incubation medium consisted of 0 25 M sucrose, 5 mM Tris, 1 mM EDTA, 2 mM $CaCl_2$, pH 7 4 and 25 mg of mitochondrial protein in a total volume of 2.5 ml The degradation was initiated by addition of 1 5 μg phospholipase A_2, purified from porcine pancreas [12] and generously supplied by Dr G H de Haas and coworkers from the Laboratory of Biochemistry, University of Utrecht, The Netherlands. Incubations were at 37 °C during different lengths of time, maximal 7 5 min The reaction was terminated by addition of EDTA to a final concentration of 4 mM. Controls were incubated in the presence of excess EDTA

After dilution of the incubation medium with sucrose–Tris–EDTA, containing 0 5 or 2 0 mg albumin per ml (bovine serum albumin, Fraction V, Sigma, St. Louis, U S.A) the mitochondrial membranes were pelleted at 250 000 $\times g$ during 30 min in a Spinco L2-65B ultracentrifuge, rotor-type Ti 60 In some experiments a wash of the

membrane material with sucrose–Tris–EDTA–albumin followed. The pellets were suspended in sucrose–Tris–EDTA–albumin.

Lipid analysis

After the incubation with phospholipase A and after the washes with sucrose–Tris–EDTA–albumin aliquots of the membrane material were extracted according to the procedure of Bligh and Dyer [13] and the lipids were separated by thin-layer chromatography using chloroform–methanol–25 % (w/v) ammonia–water (67 · 33 · 4 : 1, by vol.) as developing solvent The lipids were visualized with iodine vapor. The relevant spots were scraped off and their radioactivity contents were measured with a liquid scintillation spectrometer in 12 ml scintillation mixture, consisting of 0 7 % (w/v) PPO, 0.03 % (w/v) dimethyl-POPOP and 10 % (w/v) naphtalene in dioxane diluted five parts to one of water. Thus the extent of degradation of both phosphatidylethanolamine and phosphatidylcholine was determined as well as the efficiency with which the reaction products were removed by the albumin treatment

Enzyme assays

Succinate–cytochrome *c* reductase activity was determined by a spectrophotometric method in a total volume of 2 5 ml medium of the following composition, 0 1 M sodium phosphate buffer, pH 7 3, 0.1 M sucrose, 0 05 M KCl, 1 mM KCN, 1.5 μM rotenone, 0.08 mM cytochrome *c* (Type III, Sigma, St. Louis, U.S.A.). Membrane material was added in amounts corresponding to 0.15 mg of mitochondrial protein. The reaction was initiated by addition of sodium succinate to a final concentration of 25 mM. Under these conditions the reaction rate remained constant up to an increase in absorbance of at least 0 4 unit.

Cytochrome *c* oxidase activity was determined by the method of Smith [14] in a total volume of 3 ml medium consisting of 0.1 M sodium phosphate buffer, pH 7.3, 0 1 M sucrose, 0.05 M KCl, 1.5 μM rotenone and membrane material corresponding to 0 15 mg of protein. The reaction was started by addition of reduced cytochrome *c*, to a final concentration of approx. 0.02 mM. The rate constant of the reaction was determined by logarithmic recording of the absorbance at 550 nm

These measurements were performed with a Uvichem H1621 spectrophotometer (Hilgar and Watts, Rank Precision Industries, London, England) equipped with a thermostatted cell holder. The temperature in the cuvettes was determined with a thermocouple. At low temperatures a stream of dry air was flushed along the cuvettes to prevent condensation

RESULTS

Mitochondrial inner-membrane-matrix particles were treated with phospholipase A during different lengths of time and washed with sucrose–Tris–EDTA medium containing albumin as described above. Table I contains data on the extent of phospholipid degradation and the removal of lysophospholipids in four mitochondrial preparations (A, B, C and D). The free fatty acids were always completely removed by the albumin treatment as judged by thin-layer chromatography. The specific activities of succinate–cytochrome *c* reductase and cytochrome *c* oxidase in these preparations are also presented in Table I. Neither addition of 2,4-dinitrophenol

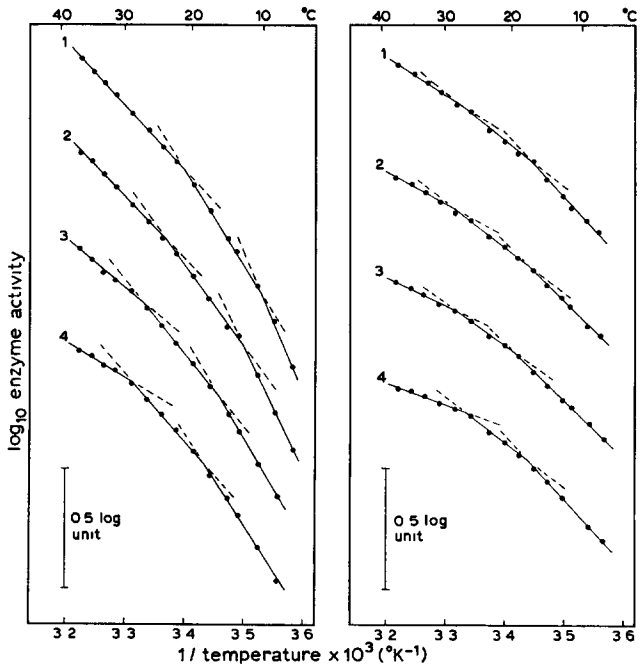
TABLE I

EXTENT OF PHOSPHOLIPID HYDROLYSIS, REMOVAL OF LYSOPHOSPHOLIPIDS AND SPECIFIC ACTIVITIES OF SUCCINATE–CYTOCHROME *c* REDUCTASE AND CYTOCHROME *c* OXIDASE IN PHOSPHOLIPASE A-TREATED INNER-MEMBRANE-MATRIX PARTICLES

The first two columns present the relative amounts of lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) in untreated and phospholipase A-treated mitochondrial inner membranes The following pair of columns give the corresponding figures after albumin treatment Preparations A, B and D were treated once with a solution containing 0 5 mg albumin per ml, with C the albumin concentration was 2 0 mg/ml and the treatment was repeated once more The specific activities of the two enzymes, measured at 25 °C, are given in the last two columns as μmoles cytochrome *c* (cyt *c*) reduced per min per mg of protein and as the first-order velocity constant (min^{-1}), related to 1 0 mg of protein, respectively Other abbreviations PE, phosphatidylethanolamine, PC, phosphatidylcholine, Succ , succinate

Prepn	Incubation time (min)	Before albumin treatment		After albumin treatment		Specific activity	
		$\frac{LPE}{PE+LPE} \times 100$	$\frac{LPC}{PC+LPC} \times 100$	$\frac{LPE}{PE+PLE} \times 100$	$\frac{LPC}{PC+LPC} \times 100$	Succ–cyt *c* reductase	Cyt *c* oxidase
A1	control	1	1	1	1	0 25	–
2	0 5	13	6	2	2	0 21	–
3	1 0	18	8	4	3	0 17	–
4	2 5	28	13	12	5	0 14	–
B1	control	1	1	–	–	0 16	4 3
2	4 0	39	17	–	–	0 13	3 1
3	6 0	51	27	–	–	0 13	2 6
4	7 5	62	31	–	–	0 16	3 7
C1	control	2	1	<1	<1	0 16	5 4
2	1 0	17	7	<1	<1	0 12	3 9
3	3 0	30	13	1	<1	0 09	2 3
4	6 0	48	22	3	1	0 16	2 0
D1	control	2	2	1	2	–	5 1
2	1 0	15	7	6	4	–	4 6
3	2 5	29	12	14	6	–	4 0
4	6 0	49	20	39	12	–	5 8

(0.1 mM) to the reaction medium nor mild sonication of the preparations resulted in any increase of the enzyme activities These findings indicate that under the conditions employed the rate-limiting steps in the assay procedures were the enzyme reactions proper The specific enzyme activities are based on the protein contents of the preparations prior to the phospholipase treatment, as this treatment causes loss of matrix protein Consequently these values have only limited accuracy, because some material may be lost during the albumin washes. Nevertheless a slight reduction of the specific enzyme activities could usually be observed after incubation with phospholipase A After relatively extensive phospholipase treatment a partial restoration of the specific activities was often detected. This suggests that the initial reduction is probably not due to irreversible inactivation but rather to suboptimal conditions created by the phospholipase treatment.



Figs 1 and 2. Arrhenius plots of succinate–cytochrome *c* reductase (Fig 1) and cytochrome *c* oxidase (Fig. 2) in phospholipase-treated and control mitochondrial inner membranes The curves presented were obtained with Preparation C Data on phospholipid hydrolysis are given in Table I under the corresponding numbers. Only the albumin-treated preparations were used. The plots are arbitrarily positioned on the vertical scale in a sequence related to the degree of phospholipid hydrolysis

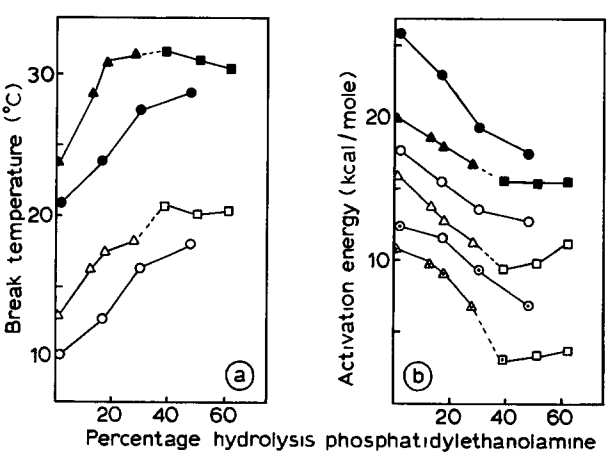


Fig. 3 Arrhenius-plot characteristics of succinate–cytochrome *c* reductase in dependence of the extent of phospholipid degradation. (a) break temperatures, (b) apparent activation energies. Triangles, Preparation A, squares, Preparation B, circles, Preparation C Additional data about these preparations are given in Table I. High- and low-temperature breaks correspond to filled and open symbols, respectively. Similarly, high, intermediate and low activation-energy values, corresponding to the three straight sections of the Arrhenius plots can be recognized by the filled, open and "dotted" symbols, respectively.

Arrhenius plots of succinate–cytochrome *c* reductase (Fig 1 presents the Arrhenius plots obtained with Preparation C) invariably revealed two distinct breaks even in control preparations, where the break temperatures were usually approx 23 and 12 °C Phospholipase treatment caused the lower-temperature break to stand out more conspicuously At the same time both breaks shifted to higher temperatures depending on the degree of phospholipid hydrolysis, but maximally to about 31 and 21 °C, respectively The apparent activation energies of the reaction gradually decrease with the extent of phospholipid degradation In Fig 3 the break temperatures and the activation energies are plotted against the extent of degradation of phosphatidylethanolamine, which corresponds to approximately twice the extent of hydrolysis of the lecithin Although the accuracy of the data is limited it is clear that the break temperatures increase with phospholipid hydrolysis, whereas the activation energies decrease Both parameters seem to reach certain limit values when approx 40 % of the phosphatidylethanolamine is hydrolyzed In Preparation C the Arrhenius plots revealed somewhat different break temperatures and activation energies than those in the Preparations A and B This can possibly be ascribed to the very effective removal of lysophospholipids by the repeated albumin treatment in this experiment On the other hand, considering the deviating values for break temperatures and activation energies in the control, animal differences might be responsible for this observation as well

Also the Arrhenius plots of cytochrome *c* oxidase (Fig 2) exhibit two discontinuous changes in slope, although the differences between the activation energies above and below the breaks are considerably smaller than in the case of succinate–cytochrome *c* reductase Fig 4 presents the dependence of the break temperatures and the apparent activation energies on the extent of phospholipid degradation There is a substantial difference with respect to this dependence between cytochrome *c* oxidase and succinate–cytochrome *c* reductase, particularly concerning the break temper-

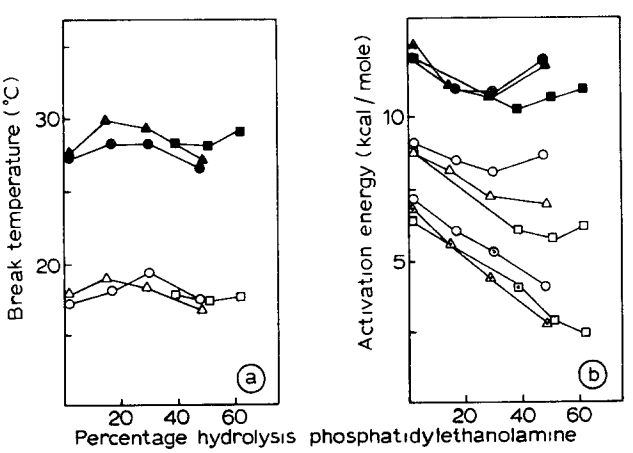


Fig 4 Arrhenius-plot characteristics of cytochrome *c* oxidase in dependence of the extent of phospholipid degradation (a) break temperatures, (b) apparent activation energies Triangles, Preparation D, squares, Preparation B, circles, Preparation C Additional data about these preparations can be found in Table I For further explanation of the symbols see legend of Fig 3

atures. In the case of succinate–cytochrome *c* reductase a considerable increase of the break temperatures was observed with phospholipid hydrolysis, whereas those of cytochrome *c* oxidase are not very strongly influenced by phospholipase treatment The data in Fig 4a are suggestive of a slight increase of the break temperatures at a low extent of phospholipid degradation and a decrease again at higher degrees of hydrolysis. However, the likely errors in determining the break temperatures from the Arrhenius plots are of the same order of magnitude as the variations presented in this figure. Also the apparent activation energies do not change very much with the extent of phospholipid hydrolysis, except the activation energy above the upper break temperature which does decrease significantly, as is shown in Fig 4b.

DISCUSSION

Phospholipid–water systems containing one pure lipid component exhibit a sharp phase change at a well-defined characteristic temperature. Mixtures of phospholipids however, usually show a broadened transition, characterized by two discrete temperatures corresponding to the beginning and end of the course of lateral phase separation [15, 16] This latter process requires, in contrast to the phase change proper, extensive lateral diffusion of the phospholipids, a phenomenon which has indeed been detected in model membranes [17, 18] as well as in biological membranes [19]. Recently strong evidence was presented for the occurrence of lateral phase separation in biological membranes [20]. Such a phenomenon is supported by data obtained by differential thermal analysis. According to calorimetric studies of Blazyk and Steim [21] rat-liver mitochondria undergo a broad thermal transition between approx −20 and +25 °C. As already mentioned by these authors sharp breaks in Arrhenius plots for physiological activities do not correlate with broad thermal transitions and cannot reflect the entire phase change. Linden et al [20] detected two breaks in Arrhenius plots for transport processes in *E. coli* membranes The break temperatures appeared to correspond to the upper and lower temperature limit of the course of lateral phase separation. The authors suggest that between those two extremes the lateral compressibility of the membranes is very much increased, thus facilitating transverse mobility of proteins involved in transport processes.

We detected two breaks in Arrhenius plots for enzyme activities The higher-temperature break seems to correspond to the upper limit of the thermal transition as detected by calorimetric methods [21], as would appear to be in accordance with findings of others [8, 22]. The lower-temperature break however, does certainly not coincide with the termination of the thermal transition. Obviously an interpretation in terms of lateral compressibility of the membrane does not seem to be suitable to explain the temperature dependence of membrane-bound enzymes. Another possible interpretation was proposed recently by Eletr et al. [23]. They concluded from their investigations that one of the two breaks in the Arrhenius plot of a membrane-associated enzyme can be due to a conformational change intrinsic to the protein In our study however, the Arrhenius diagrams exhibit lipid dependence which was most pronounced in the case of succinate–cytochrome *c* reductase. The reaction catalyzed by succinate–cytochrome *c* reductase is a multi-step process. For this enzyme the occurrence of the lower-temperature break can possibly be explained by different rate-limiting steps above and below this temperature. Obviously such an

explanation would not be valid for cytochrome *c* oxidase We propose therefore the following interpretation of the occurrence of two breaks in Arrhenius plots of membrane-associated enzymes

Below the higher temperature break, usually interpreted as reflecting the phase transition of the membrane lipids [5–8, 22, 24], the lipids directly influencing the conformation of the enzyme molecules are in a gel phase Upon cooling below this break temperature the composition of the gel phase continuously changes Recent studies on model systems indicate that reorganizations in the gel phase may occur due to immiscibility of the lipid components in this phase [16, 25] A similar reorganization could possibly occur in biological membranes either locally or in the entire gel phase If the lipid molecules determining the conformational state of the enzyme are involved in such a reorganization a change in the activation energy of the enzyme reaction could result. For changes in the gel phase to occur lateral motion of the phospholipids in this phase is required According to Shimshick and McConnell [16] the lateral diffusion of the phospholipids in the gel phase may indeed be very high Obviously, the possibility cannot be excluded that the lower-temperature break is related to another "liquid-gel" phase transition involving lower melting lipids

It is likely that during the course of the lateral phase separations the lipids are not randomly distributed in the membrane [26]. However, also at high temperatures, when the lipids are all in the liquid-crystalline phase some lipid heterogeneity may exist due to the presence of proteins This could result in a partial solidification of the lipids surrounding enzyme molecules when the temperature is lowered The assumption that the lipids which influence the conformation of the enzyme molecules are in a gel phase below the higher break temperature does not require, therefore, that the entire enzyme molecule be in the gel phase. The fact that the higher-temperature breaks are observed in the most upper region of the thermal transition detected by differential scanning calorimetry may indicate that the more saturated phospholipid species specifically influence the conformation of the enzyme molecules Referring to the relatively high transition temperatures of phosphatidylethanolamines as compared to lecithins [16] of identical fatty acid composition the specific involvement of the former class of phospholipids should also be kept under consideration Phospholipase action on mitochondrial membranes results in a drastic modification of the relative proportions of phospholipid classes as well as molecular species [27] This can explain the influence of the extent of phospholipid degradation on the break temperatures in the Arrhenius plots of the enzyme activities studied. The gradual alteration of the apparent activation energies as a result of phospholipase treatment indicates that the enzyme reactions are influenced not only by the physical state of the membrane lipids but also in a direct manner by the very composition of this lipid environment By contrast, the activation energies of transport processes do not seem to be very strongly dependent on the composition of the lipid bilayer [1, 2, 4] This supports the idea of Linden et al. [20] that transport processes are influenced by the membrane lipids in quite another way than membrane-bound enzyme activities

The observation that the two enzyme activities studied differ with respect to the dependence of break temperatures and activation energies on phospholipase treatment suggests that there may exist a certain degree of heterogeneity within the lipid phase of the mitochondrial inner membrane

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