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INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON MITOCHONDRIAL MEMBRANES

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Summary

Mitochondrial phospholipids from goldfish lateral line muscle were analysed with respect to polar and apolar groups. Groups of 20 goldfish, acclimated to 5, 20 and 30°C, were used. Temperature-induced shifts of both polar and apolar groups of the mitochondrial phospholipids were observed. The fatty acid composition of mitochondrial phospholipids is characterized by a large amount of polyenoic acids, dominated by docosahexaenoic acid and by octadecadienoic acid. At the higher acclimation temperatures, a significant decrease in docosahexaenoic acid is found. However, the resultant effect of environmental temperature on the degree of unsaturation is small, in contrast to the marked effect on mean chain length. Pronounced changes in the molar ratio of phosphatidylcholine and phosphatidylethanolamine are seen; a decrease in mitochondrial phosphatidylcholine is observed at low acclimation temperature, which is compensated for by a nearly equal increase in phosphatidylethanolamine. The main phospholipids are, apparently, phosphatidylcholine, phosphatidylethanolamine and cardiolipin, comprising 90% of the total pool of 12 species. It is found that the anionic nature of the phospholipids is increased at low acclimation temperatures. We discuss this effect and its probable importance in the stabilization of the surface potential of the mitochondrial membranes.

Introduction

Many organisms have adaptive mechanisms with respect to environmental changes, which apparently support homeostasis of the functions of life. A

general response of poikilothermic organisms to a changed environmental temperature is the inverse shift in the degree of unsaturation of membrane lipids [1]. In biomembrane research, several studies on micro-organisms demonstrated a correlation between thermotropic lipid phase changes and breaks in Arrhenius plots of membrane-bound enzymes. The transition temperature appears to be correlated to the ratio of unsaturated to saturated membrane lipids, which controls membrane fluidity [2]. Incorporation of a greater proportion of *cis*-unsaturated carbon-carbon bonds is often presumed to be the means by which organisms adapt to a change in environmental temperature [1—8]; however, not only lipids but also proteins have been found to influence the membrane fluidity [9]. In addition, it has been observed that incorporation of membrane proteins is dependent on the degree of unsaturation of membrane lipids [10], so a certain part of the unsaturated lipids may be bound in lipid-protein complexes. In a review, Sandermann [11] discusses the complexity of the viscotropic effects of membrane lipids and the indistinctness of their role in membrane functioning due to specific protein-lipid interactions. Apparently, the adaptive response to changed environmental temperature can be very complex, especially in membranes characterized by a high protein-to-lipid ratio, such as mitochondrial membranes.

In a previous study on goldfish mitochondria [12], it has been shown that discontinuities in Arrhenius plots of state III respiration were found in preparations of red muscle, white muscle and liver. Adaptation to 5, 20 and 30° C resulted in a shift of the break temperature, giving low activation energies at the adaptation temperature. Analysis of the fatty acid composition of the same preparations demonstrated no consistent change in degree of unsaturation due to changed environmental temperature. Not only apolar chains but also polar head groups of phospholipids can have marked effects on membrane fluidity [13,14]; specific phospholipids can modulate membrane-bound enzymes, so a change in phospholipid composition might be an important factor in the homeostasis of membrane functioning. In this study, we analysed the apolar as well as the polar part of phospholipids extracted from lateral line muscle mitochondria of goldfish acclimated to 5, 20 and 30° C.

Materials and Methods

Conditions. Healthy goldfish weighing about 100 g were kept at 20° C for at least 2 months; three groups of 20 fish were randomly selected and acclimated to 5, 20 and 30° C, respectively. The acclimation levels were reached by a daily temperature change of 1.0° C; the fish were kept at the final temperature for at least 6 weeks in tanks with air-saturated running tap water. Food composition has been described earlier [12].

Preparation of mitochondria. Mitochondria were isolated from lateral line muscles of two fishes at a time according to the method described by van den Thillart and Modderkolk [12]. The mitochondrial pellets were resuspended in 4 ml isolation medium with 0.2% bovine serum albumin. After freeze-drying, the mitochondria suspensions, each from four fish, were stored under N_2 at —35° C and analysed within 6 weeks.

Lipid analysis. Lipid extractions and analyses were performed in an N_2-

flushed atmosphere. Freeze-dried mitochondria were extracted three times with, respectively, 60, 50 and 40 ml $CHCl_3/CH_3OH$ (2 : 1, v/v). Proteolipid complexes were broken by repeated evaporation of water-wet $CHCl_3$ [15], and water-soluble components were removed according to the method of Folch et al. [16]. Aliquots of lipid extracts were separated by two-dimensional thin-layer chromatography on 20 × 20 cm plates coated with 300 μm Silica gel HR (Merck) containing 9% magnesium acetate [17—19].

Phospholipids were separated from neutral lipids and saponified in 4 ml of 1 M KOH (80% CH_3OH) at 65°C for 90 min. Fatty acids were extracted after acidifying to pH 1 with petroleum ether (40 : 60, v/v) and methylated according to the method of Kluytmans [20]. Methyl esters were analysed using a Becker-409 gas chromatograph equipped with a flame ionization detector and coupled to an Autolab 6300 digital integrator. Separation was performed on a 16 m W.C.O.T. column at 180°C with Carbowax 20 M as stationary phase. Identification was achieved by comparison with Supelco standards, by graphical interpolation methods and by hydrogenation of unsaturated fatty acid methyl esters [21].

Results and Discussion

Mitochondria were isolated from the lateral line muscles of goldfish acclimated to 5, 20 and 30°C. The extraction data (Table I) show a significant reduction in the yield of phospholipids and proteins at 30°C, which apparently correlates with the low oxidative capacity of the tissues at high acclimation temperatures [12,23]. The ratio of phospholipid to protein appears to be slightly temperature dependent; our data are in agreement with those for porcine heart [24] and carp liver [4] mitochondria.

The influence of environmental temperature on the phospholipid composition of poikilotherms is described by several authors [3,4,7,8,25—29]; how-

TABLE I

QUANTITATIVE EXTRACTION DATA OF MITOCHONDRIAL SUSPENSIONS, PREPARED FROM GOLDFISH LATERAL LINE MUSCLE

The data indicate mean ±S.D., followed by the number of observations in brackets. The level of significance (*P*) is measured according to the Student's *t*-test. Phospholipids are determined colorimetrically as phosphate after previous purification on Silica gel HR. Protein was estimated according to the method of Schackterle and Pollack [22].

Mitochondrial parameters	Acclimation temperature (°C)			*P*(%)		
	5	20	30	5—20°C	5—30°C	20—30°C
mg wet wt./g muscle	53.3 ± 8.4 (n = 10)	55.1 ± 10.4 (n = 10)	38.6 ± 6.6 (n = 10)	—	<1	<1
mg phospholipid/g muscle	2.30 ± 0.39 (n = 4)	1.96 ± 0.23 (n = 5)	1.09 ± 0.16 (n = 5)	—	<1	<1
mg protein/g muscle	7.30 ± 1.52 (n = 4)	7.05 ± 0.92 (n = 5)	5.20 ± 1.53 (n = 5)	—	—	<5
mg phospholipid/mg protein	0.33 ± 0.03 (n = 4)	0.28 ± 0.02 (n = 5)	0.22 ± 0.05 (n = 5)	<5	<1	<5

TABLE II

PHOSPHOLIPID COMPOSITION OF LATERAL LINE MUSCLE MITOCHONDRIA FROM GOLDFISH ACCLIMATED TO 5, 20 and 30°C

The data represent mean ±S.D. of phospholipid phosphorus as a percentage of total recovery. The level of significance, P measured according to the Student's t-test is based on the number (n) of different extractions, which were analysed in duplicate. Separation was achieved by two-dimensional TLC according to the method of Rouser et al. [17]. In each group, 20 fishes were used. n.d., not detectable.

Phospholipid species	Acclimation temperature (°C)			P (%)		
	5 (n = 4)	20 (n = 5)	30 (n = 3)	5—20°C	5—30°C	20—30°C
Phosphatidylcholine	43.70 ± 1.53	48.14 ± 1.57	56.90 ± 2.21	<1	<0.1	<0.1
Phosphatidylethanolamine	39.30 ± 2.17	33.97 ± 1.28	24.92 ± 1.43	<1	<0.1	<0.1
Cardiolipin	10.09 ± 0.76	10.85 ± 0.13	8.22 ± 0.92	<5	<5	<0.1
Phosphatidylinositol	2.80 ± 0.12	2.73 ± 0.10	3.07 ± 0.35	—	—	—
Phosphatidylserine	0.78 ± 0.35	0.69 ± 0.12	1.32 ± 0.36	—	—	1
Sphingomyelin	0.26 ± 0.05	0.37 ± 0.12	1.70 ± 0.42	—	<0.1	<0.1
Phosphatidic acid	0.16 ± 0.12	n.d.	n.d.	<0.1	<0.1	—
Phosphatidylglycerol	0.45 ± 0.06	0.60 ± 0.10	0.54 ± 0.07	<5	—	—
Lysophosphatidylcholine	0.68 ± 0.09	1.32 ± 0.32	1.24 ± 0.42	<1	<5	—
Lysophosphatidylethanolamine	0.53 ± 0.11	0.69 ± 0.20	0.40 ± 0.17	—	—	—
Lysobisphosphatidic acid	1.06 ± 0.20	0.28 ± 0.07	0.90 ± 0.25	<1	—	1
Unidentified	0.19 ± 0.05	0.35 ± 0.13	0.56 ± 0.30	—	—	—

ever, only a few analysed more than four different kinds of phospholipid. In Table II the phospholipid composition of mitochondria isolated from 5-, 20- and 30°C-acclimated fish is summarized. About 12 phosholipid species have been determined, three of which comprise about 90% of the total pool, i.e., phosphatidylcholine, phosphatidylethanolamine and cardiolipin. The content of both phosphatidylcholine and phosphatidylethanolamine is found to be strongly dependent on the acclimation temperature. As the content of these compounds changes in opposite directions, the ratio of phosphatidylcholine to phosphatidylethanolamine ranges from 1.1 to 2.3. Significant shifts in other phospholipids are less pronounced. Cardiolipin levels are lowered at 30°C; phosphatidylserine, sphingomyelin and lysophosphatidylcholine levels are raised at 30°C. It is interesting that phosphatidic acid occurs at 5°C but is absent at 20 and 30°C.

Only minor shifts of lysoglycerophospholipids occurred in goldfish mitochondria, in contrast to those in squirrel heart mitochondria [18]. At low temperatures, hibernating squirrels incorporate lysoglycerophospholipids in order to stabilize membrane fluidity. Apparently, this kind of low temperature adaptation does not occur in goldfish. However, dissimilar results might have been expected, for hibernating mammals survive low temperatures in a torpid state, while cold-acclimated goldfish change their activity very litte.

The preference for ethanolamine to choline in phospholipids of cold-acclimated poikilothermic animals is described by several authors [3,4,8,25,27]; the most striking effects, however, can be observed in purified membranes, in contrast to phospholipids extracted from whole organs. From several studies, it appears that the phospholipid pattern is highly dependent on the subcellular fraction [24,30]. Therefore, it can be expected that adaptational responses cannot always be recognized when whole tissue extracts are analysed. The inverse relationship between acclimation temperature and the mitochondrial phosphatidylethanolamine content is still unclear. The small polar head group allows very close packing and low water penetration [31], thus reducing permeability and fluidity. For example, enzymatic methylation of phosphatidylethanolamine in erythrocyte ghosts [14] results in a decreasing viscosity of the membrane. Apparently, incorporation of phosphatidylethanolamine in biomembranes during cold acclimation is not a homeoviscosity response; on the contrary, it seems to have the opposite effect, although the large difference between the phase-transition temperatures of phosphatidylethanolamine and phosphatidylcholine can be reduced by incorporation of *cis*-unsaturated fatty acids [13]. At a neutral pH, phosphatidylethanolamine is negatively charged, whereas the net charge of phosphatidylcholine is zero [32,33]. The replacement of choline by ethanolamine, therefore, may be a way to increase the anionic nature of the membrane. This phenomenon can be explained as a means to stabilize the membrane surface potential. A surface potential is necessary to prevent aggregation of small particles. The negative charge on biomembranes is caused by partial dissociation of acidic groups in proteins and phospholipids. Dissociation of these groups, however, is temperature dependent, primarily due to the fact that aqueous dissociation is strongly temperature dependent. The general dependency is given by the van't Hoff relationship: $d\ln K/dT = H/RT^2$. The influence of temperature

decrease on the pK value of weak acidic groups cannot be neglected; in general, a 2% decrease is found when the temperature is lowered by 10°C. So a decrease in temperature will lower the surface potential of biomembranes. Therefore, it is probable that the observed increase in the anionic nature of the biomembrane at low acclimation temperatures is necessary in the stabilization of the surface charge. This hypothesis is also supported by the fact that phosphatidic acid, a strongly negatively charged compound, was found at 5°C, but not at 20 or 30°C. The content of phosphatidylserine, a negatively charged compound, is higher at 30°C than at 20 or 5°C. The decrease in phosphatidylserine from 30 to 20°C, however, was overcompensated by an increase in cardiolipin, which must be regarded to be also negatively charged.

The fatty acid composition of the mitochondrial phospholipids is presented in Table III. Approx. 50 compounds were detected in each sample, of which 29 are at levels above 0.1%, 14 above 1% and only six above 5%. The latter are the fatty acids, 16 : 0, 18 : 0, 18 : 1, 18 : 2, 20 : 4 and 22 : 6, making up about 70% of the total pool. Considering the influence of an increased acclimation temperature on the fatty acid composition, we found statistically positive shifts in the concentrations of 15 : 0, 16 : 1, 17 : 1, 18 : 0, 18 : 1 and 18 : 2 and negative shifts in the concentrations of 20 : 1, 20 : 2, 20 : 4, 22 : 2, 22 : 4, 22 : 5 and 22 : 6. This clearly demonstrates that acclimation temperature has a primary effect on both chain length and degree of unsaturation. The main shifts were found in the concentration of 18 : 1, 18 : 2 and 22 : 6; we found, respectively, +2.7, +2.3 and −3.7% due to an increased environmental temperature from 5 to 30°C.

In general, it can be concluded from fatty acid analysis of different phospholipid species that interpretation of data with regard to a change of environmental temperature is very difficult [3—5,24,27]. The general trend is towards polyunsaturated long-chain fatty acids at reduced environmental temperature; this trend can also be observed in phospholipids from goldfish lateral line muscle mitochondria (Table IV). No change can be observed in the total amount of saturated fatty acids (primarily 16 : 0 and 18 : 0), in contrast with mono- and polyenoic unsaturated fatty acids. The main polyenoic fatty acids are 18 : 2, 20 : 4 and 22 : 6; at the lower temperatures the concentration of 18 : 2 decreases, that of 22 : 6 increases and the concentration of 20 : 4 remains the same. Obviously, there is a marked tendency to incorporate long-chain fatty acids at reduced temperatures, so that at 5°C the mean chain length is 20.1 and 30°C, 17.7 carbon atoms. Farkas et al. [34] demonstrated that incorporation of long-chain fatty acids in liver phospholipids of carp was favored at low temperatures irrespective of the acclimation temperature. The rate of production of saturated fatty acids remains at a low level in the cold, while that of long-chain polyunsaturated ones increases gradually from 13 to 61%. This process might explain differences in the degree of unsaturation of membrane lipids of poikilotherms, as found by various authors [4—8,12, 26,27,34]. The length of the acclimation period determines the amount of the incorporated unsaturated fatty acids.

In goldfish muscle mitochondria, we found significant changes in fatty acid families. The oleic acid (18 : 1, (9) *) family increased from 11.7% at 5°C

* The number in parentheses represents the omega counting of the first carbon-carbon double bond.

TABLE III

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS ISOLATED FROM LATERAL LINE MUSCLE MITOCHONDRIA

Data represent mean of mole percentages ±S.D.; the level of significance (*P*) between groups, measured according to the Students' *t*-test, is based on the number (*n*) of different preparations which were analysed in duplicate. In each group, 20 fishes have been used. Phospholipids were purified by thin-layer chromatography and saponified. Methylated fatty acids were analysed on an open capillary column (Carbowax 20M) at 180°C. Components less than 0.1% have been omitted. i, iso-I; ai, *anti*-I.

Fatty acid	Acclimation temperature (°C)			*P* (%)		
	5 (*n* = 5)	20 (*n* = 5)	30 (*n* =2)	5—20°C	5—30°C	20—30°C
14 : 0	0.76 ± 0.40	0.87 ± 0.13	1.26 ± 0.03	—	—	<1
15 : 0 i	0.07 ± 0.04	0.11 ± 0.02	0.10 ± 0.02	—	—	—
15 : 0 ai	0.11 ± 0.05	0.15 ± 0.04	0.23 ± 0.06	—	—	—
15 : 0	0.38 ± 0.13	0.42 ± 0.06	0.49 ± 0.01	<2	<1	—
16 : 0 i	0.07 ± 0.04	0.07 ± 0.01	0.07 ± 0.01	—	—	—
16 : 0 ai	0.38 ± 0.13	0.38 ± 0.14	0.90 ± 0.75	—	—	—
16 : 0	12.56 ± 1.02	11.92 ± 1.10	13.17 ± 0.40	—	—	—
16 : 1 (9)	2.23 ± 0.27	2.61 ± 0.13	2.60 ± 0.24	<2	—	—
16 : 2 (6)	0.15 ± 0.07	0.08 ± 0.03	0.07 ± 0.01	—	—	—
17 : 0 i	0.59 ± 0.03	0.56 ± 0.10	0.57 ± 0.04	—	—	5
17 : 0 ai	0.22 ± 0.10	0.17 ± 0.03	0.23 ± 0.02	—	—	—
17 : 0	0.74 ± 0.08	0.65 ± 0.06	0.69 ± 0.04	—	—	—
17 : 1 (8)	0.49 ± 0.13	0.61 ± 0.04	0.70 ± 0.01	—	—	<5
17 : 2	0.18 ± 0.07	0.11 ± 0.03	0.20 ± 0.14	—	—	—
18 : 0 i	0.33 ± 0.06	0.33 ± 0.08	0.58 ± 0.08	—	<1	<2
18 : 0 ai	0.05 ± 0.02	0.02 ± 0.02	0.04 ± 0.01	—	—	—
18 : 0	8.24 ± 0.16	8.11 ± 1.00	8.45 ± 0.60	—	—	—
18 : 1 (9)	7.11 ± 0.37	8.23 ± 0.72	14.87 ± 0.33	<2	<1	<1
18 : 1 (6)	5.13 ± 0.40	5.24 ± 0.21	0.00	—	<1	<1
18 : 2 (6)	12.76 ± 1.06	15.43 ± 0.48	15.10 ± 1.21	<1	5	—
18 : 3 (6)	2.52 ± 0.23	2.54 ± 0.27	2.48 ± 0.98	—	—	—
19 : 0 i	0.13 ± 0.03	0.10 ± 0.03	0.03 ± 0.03	—	1	5
19 : 0 ai	0.16 ± 0.04	0.26 ± 0.13	0.14 ± 0.03	—	—	—
19 : 0	0.62 ± 0.13	0.61 ± 0.08	0.48 ± 0.05	—	—	—
20 : 0 i	0.22 ± 0.06	0.22 ± 0.04	0.27 ± 0.01	—	—	—
20 : 0 ai	0.15 ± 0.08	0.08 ± 0.03	0.10 ± 0.04	—	—	—
20 : 0	0.42 ± 0.03	0.45 ± 0.06	0.36 ± 0.01	—	<5	—
20 : 1 (9)	2.35 ± 0.23	2.41 ± 0.16	1.71 ± 0.24	—	2	<1
20 : 1 (6)	0.19 ± 0.04	0.17 ± 0.07	0.16 ± 0.04	—	—	—
20 : 2 (6)	1.26 ± 0.13	1.31 ± 0.08	1.11 ± 0.10	—	—	<5
20 : 3 (6)	1.21 ± 0.18	1.29 ± 0.19	1.11 ± 0.05	—	—	—
20 : 3 (3)	0.47 ± 0.10	0.47 ± 0.18	0.29 ± 0.11	—	—	—
20 : 4 (6)	6.95 ± 0.92	7.23 ± 0.74	7.79 ± 0.31	—	—	—
20 : 4 (3)	0.60 ± 0.11	0.46 ± 0.05	0.31 ± 0.04	<5	<2	<2
20 : 5 (3)	4.47 ± 0.86	3.67 ± 0.55	2.86 ± 0.31	—	5	—
20 : 6 (3)	0.25 ± 0.04	0.42 ± 0.28	0.34 ± 0.03	—	5	—
22 : 0	0.07 ± 0.02	0.11 ± 0.04	0.07 ± 0.03	—	—	—
22 : 2	1.28 ± 1.51	0.29 ± 0.18	0.23 ± 0.07	<5	<5	—
22 : 4 (6)	1.60 ± 1.41	0.93 ± 0.29	0.93 ± 0.35	—	—	—
22 : 5 (6)	1.61 ± 0.19	1.60 ± 0.21	1.43 ± 0.08	—	—	—
22 : 5 (3)	2.99 ± 0.49	2.42 ± 0.23	2.13 ± 0.07	<5	—	—
22 : 6 (3)	17.25 ± 0.52	15.86 ± 1.30	13.55 ± 0.22	5	<0.1	5
24 : 1	0.14 ± 0.08	0.17 ± 0.09	0.16 ± 0.23	—	—	—

TABLE IV

INFLUENCE OF ACCLIMATION TEMPERATURE ON THE ACYL CHAINS OF PHOSPHOLIPIDS ISOLATED FROM GOLDFISH LATERAL LINE MUSCLE MITOCHONDRIA

Data, derived from original figures underlaying Table III, indicate mean ±S.D. Mole percentages of fatty acid were summated in three classes: saturated, monoenoic and polyenoic. The unsaturation index is defined by $\Sigma\ m_i \cdot n_i$, where m_i is the mole percentage and n_i the number of carbon-carbon double bonds of fatty acid i. The mean chain length is defined by $\Sigma\ f_i \cdot c_i$, where f_i is the mole fraction and c_i the number of carbon atoms of fatty acid i. The significance of differences (P) is measured according to the Students' t-test, based on the number (n) of different extractions. At each acclimation temperature, 20 goldfish were used.

Acclimation temperature	Summated fractions			Unsaturation index	Chain length
	Saturated	Monoenoic	Polyenoic		
5°C (n = 4)	26.23 ± 1.7	18.14 ± 0.7	55.63 ± 2.1	249.5 ± 9.3	20.09 ± 0.07
20°C (n = 5)	25.79 ± 1.7	20.12 ± 0.9 *	54.09 ± 0.3	239.6 ± 11.4	18.62 ± 0.08 *
30°C (n = 2)	28.41 ± 0.3	21.61 ± 0.1 **	49.98 ± 1.0 **	219.6 ± 4.1 **	17.72 ± 0.02 **

* $P < 0.01$ comparing 5 with 20°C series.
** $P < 0.01$ comparing 5 with 30°C series.

to 19.2% at 30°C, primarily due to changes in oleic acid content. The linoleic acid (18 : 2 (9)) family had a maximum at 20°C correlated with a decrease in the linoleic acid content at 5°C and a disappearance of *cis*-12-octadecaneoic acid at 30°C. The linolenic acid (18 : 3 (3)) family changed from 19.5% at 30°C to 26.0% at 5°C, caused by a unidirectional shift in icosapentaenoic and docosahexaenoic acid content.

Thus, the maximal level of the oleic family is found at 30°C, of the linoleic family at 20°C and of the linolenic family at 5°C. Similar results were observed in trout liver [3]. In carp liver mitochondria, an increase in the linoleic acid family was found at the lower temperature; however, the experiments were performed at 10 and 26°C and it is possible that incorporation of the linolenic acid family would occur at temperatures below 10°C.

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