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TEMPERATURE ADAPTATION OF BIOLOGICAL MEMBRANES

COMPENSATION OF THE MOLAR ACTIVITY OF CYTOCHROME *c* OXIDASE IN THE MITOCHONDRIAL ENERGY-TRANSDUCING MEMBRANE DURING THERMAL ACCLIMATION OF THE CARP (*CYPRINUS CARPIO* L.)

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Summary

The acclimation temperature of carp does not affect the amount of cytochrome *c* oxidase per mg mitochondrial protein as revealed from the reduced-minus-oxidized difference spectra of red muscle mitochondria from cold- and warm-acclimated carp.

There are no differences between cold- and warm-acclimated fish in the substrate binding properties of the enzyme as judged from the K_m values for cytochrome *c* at 30°C (3.34 ± 0.33 μM, acclimation temperature 10°C and 3.55 ± 0.31 μM, acclimation temperature 30°C).

The molar activities of the enzyme, however, differ for both acclimation temperatures: when intercalated in the 10°C-acclimated mitochondrial membrane, the enzyme can catalyze the oxidation of 117.6 ± 17.2 mol ferrocytochrome *c*/s per mol heme *a* as compared with 85.6 ± 17.2 in the 30°C-acclimated membrane (experimental temperature 30°C).

Correspondingly, higher specific activities of the succinate oxidase system are observed in mitochondria from cold-acclimated carp as compared with those obtained from warm-acclimated carp.

The results indicate that cold acclimation of the eurythermic carp is accompanied by a partial compensation of the acute effect of decreasing temperature on the activity of cytochrome *c* oxidase in red muscle mitochondria.

Based on the temperature-induced lipid adaptation reported for carp red muscle mitochondria (Wodtke, E. (1980) Biochim. Biophys. Acta 640, 698—709), it is concluded that during thermal acclimation the molar activity of

cytochrome *c* oxidase is controlled by viscotropic regulation. The results fit to the conception that cardiolipin constitutes a lipid shell (annulus) surrounding the oxidase within the native membrane, but that it is the bilayer fluidity and not the annular fluidity which determines the activity of cytochrome *c* oxidase.

Introduction

During the acclimation to changed environmental temperatures, the physiology and metabolism of poikilothermic vertebrates are often controlled in a manner that keeps the energy turnover of the animal partly independent of ambient temperature (for a general treatment, see the papers in Precht et al. [1]). Increased activities of mitochondrial membrane-bound enzymes during cold acclimation frequently have been shown to be involved in these adaptive processes [2—7]. According to the general observation that lipid unsaturation often correlates inversely with acclimation temperature, it was reasonable to suppose that acclimation temperature-dependent activity changes may, at least in part, reflect viscotropic [8] regulation of enzyme activity. This hypothesis has been examined in work with mitochondria of thermally acclimated goldfish in which cytochrome *c* oxidase [9] and succinic dehydrogenase [10] were studied. One of these communications, however, lacks a fatty acid analysis [10] whereas the other [9] reports fatty acid profiles for total lipid extracts only. The conclusions were contradictory.

In the preceding paper [11], it was demonstrated for carp red muscle mitochondria that cold environmental temperature dramatically increases the unsaturation of the neutral phosphatidylcholine, but markedly decreases unsaturation of the negatively charged cardiolipin. This has been interpreted to indicate a cold-induced increase in mitochondrial bilayer fluidity concomitant with an adjustment of specific cardiolipin-protein interactions at the decreased temperature. It is thus supposed that the molar activity of cytochrome *c* oxidase in these mitochondria might be controlled via viscotropic regulation during thermal acclimation.

This paper reports studies on the activity of cytochrome *c* oxidase and on the cytochrome concentrations in red muscle mitochondria of carp acclimated to low (10°C) and high (30°C) environmental temperatures. The results might be of general interest with respect to the role of phospholipid in the activity control of cytochrome *c* oxidase.

Materials and Methods

Animals. Carp (*Cyprinus carpio* L., 800—1200 g) were obtained from the Bundesforschungsanstalt für Fischerei, Ahrensburg/Schl.-H., and kept in aerated tap water at 20 ± 1°C. Fish were acclimated during summer to warm (30 ± 0.5°C) and cold (10 ± 0.5°C) temperatures for at least 30 days under a natural photoperiod (15 h light at the date of killing). Food (trout pellets, type 35, Ströh) was given ad libitum.

Chemicals. Horse heart cytochrome *c*, type III and tetramethyl-*p*-phenylenediamine were obtained from Sigma GmbH (Munich); bovine serum albumin,

crystallized, from Serva (Heidelberg). All other salts and reagents were purchased from Merck AG (Darmstadt) and were analytical or biochemical purposes graded.

Tissue fractionation. Mitochondria, containing the fraction between $700 \times g \cdot 10$ min and $6000 \times g \cdot 10$ min, were isolated from red superficial muscle essentially as described in the preceding study [11]. Mitochondria were frozen in 100-μl aliquots in liquid N_2 and stored for up to 3 weeks.

Analytical procedures. Mitochondria which were freeze-thawed only once were used for the enzyme assays and cytochrome spectra.

Enzyme assays. The polarographic assay of cytochrome *c* oxidase activity was carried out at 10 and 30°C using a Clark-type oxygen electrode (Eschweiler, Kiel) in a thermostatically controlled glass chamber (1.2 ml) containing cytochrome *c*, 0.333 mM tetramethyl-*p*-phenylenediamine, 3.33 mM ascorbate, and buffer I (225 mM mannitol, 75 mM sucrose, and 50 mM Tris base, neutralized to pH 7.5 with HCl). The reaction was started with 50 μl of mitochondria thawed and diluted 10-fold with buffer I no more than 60 min prior to the assay (60—100 μg protein). The observed rates were corrected for the cytochrome *c*/ascorbate/tetramethyl-*p*-phenylenediamine autooxidation. The saturation concentrations of O_2 in the air-saturated buffer I are 7.29 ngatom/ml at 10°C and 4.36 ngatom/ml at 30°C [12]. Evaluation of the kinetic parameters was accomplished by calculating the Eadie-Hofstee linearization of the saturation curves using the Hewlett Packard calculator 5805 A.

The polarographic assay of the succinate oxidase system was carried out at 10 and 30°C following succinate activation essentially as described earlier [13].

Cytochrome spectra. The cytochrome composition of the mitochondrial suspensions were determined from the α-peaks of the reduced-minus-oxidized difference spectra using the UV-300 spectrophotometer (Shimadzu). Mitochondria were diluted with buffer I to a concentration of approx. 2 mg protein/ml. Having established the baseline between 650 and 500 nm, the contents of the sample cell were reduced with a few grains of $Na_2S_2O_4$ and the difference spectra were recorded immediately and repeated at 3, 6 and 9 min following reduction. Usually, the spectrophotometer/recorder was operated in the range of ±0.025 absorption unit per full scale deflection, scan speed 75 nm/min, scale 10 nm/cm. The following wavelength couples and millimolar extinction coefficients were used: cytochrome aa_3, 605—630 nm, $\epsilon_{mM} = 24.0$ [14]; cytochrome *b*, 562—575 nm, $\epsilon_{mM} = 20.0$ [15]; cytochrome $c + c_1$, 551—540 nm, $\epsilon_{mM} = 19.1$ [16].

Protein determination. Protein was determined by a biuret method which corrects for turbidity by KCN decolorization [17]. Bovine serum albumin was used as standard.

Statistics. The Student's *t*-test was employed to test for significant differences between the means of data.

Results

Kinetic studies

Cytochrome c oxidase. Fig. 1a shows typical hyperbolic saturation curves of cytochrome *c* oxidase activity as obtained at 30°C with freeze-thawed

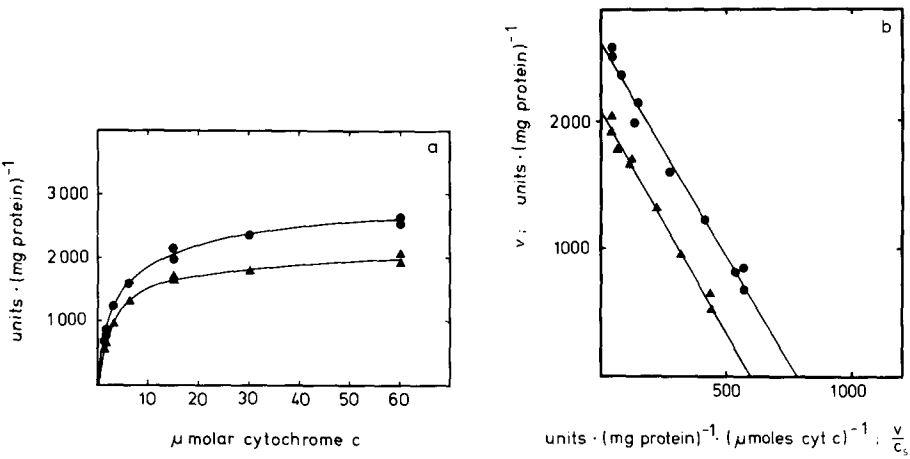

Fig. 1. (a) Comparison of the activities of cytochrome *c* oxidase in mitochondria from cold- and warm-acclimated carp with increasing concentrations of cytochrome *c* at 30°C. Cold-acclimated, 10°C (●); 51.3 μg protein/ml, 44 nM cytochrome aa_3. Warm-acclimated, 30°C (▲); 51.0 μg protein/ml, 45 nM cytochrome aa_3. More details are given in Materials and Methods. (units = nmol ferrocytochrome *c* oxidized per min). (b) Eadie-Hofstee plots of cytochrome *c* oxidase activities for cytochrome *c*. Conditions as in a. The straight lines represent the calculated equations, $y = -3.31x + 2598$ (●) (cold-acclimated) and $y = -3.44x + 2064$ (▲) (warm-acclimated).

mitochondria from red muscle of carp acclimated to cold and warm environmental temperatures. The corresponding Eadie-Hofstee plots (Fig. 1b) are linear for the cytochrome *c* concentrations applied, ranging from 30 to 1.5 μM. The maximum specific activity is higher for the enzyme from carp acclimated to 10°C as compared with 30°C. The affinity for cytochrome *c*, however, is not significantly affected by the thermal history of the animals. K_m values at 30°C obtained from five mitochondrial preparations of cold- and warm-acclimated fish are 3.34 ± 0.33 μM (acclimation temperature 10°C) and 3.55 ± 0.31 μM (acclimation temperature 30°C).

Table I gives a summary of the specific activities (V) of cytochrome *c* oxidase in mitochondria of both acclimation groups, each assayed at 10 and

TABLE I

THE INFLUENCE OF ACCLIMATION TEMPERATURE AND ASSAY TEMPERATURE ON THE ACTIVITY OF CYTOCHROME *c* OXIDASE IN CARP RED MUSCLE MITOCHONDRIA

Data indicate mean ± S.D. of maximum enzyme activity obtained from linear regression analysis of Eadie-Hofstee plots with 8—11 datum points each. For the assay composition see Materials and Methods. The level of significance (P) is based on five preparations (n = 5) in both acclimation groups. n.s., not significant. Cytochrome *c* oxidase activity is expressed as nmol cytochrome *c*/mg protein per min.

Acclimation temperature	Cytochrome *c* oxidase activity		Q_{10}
	10°C	30°C	
10°C (n = 5)	1412 ± 252	3003 ± 587	1.46 ± 0.04
30°C (n = 5)	1096 ± 132	2230 ± 313	1.43 ± 0.02
P	<0.05	<0.05	n.s.

TABLE II

THE INFLUENCE OF ACCLIMATION TEMPERATURE AND ASSAY TEMPERATURE ON THE ACTIVITY OF THE SUCCINATE OXIDASE SYSTEM IN CARP RED MUSCLE MITOCHONDRIA

Data indicate mean ± S.D. of optimum activities of the succinate oxidase system. Assays were performed in triplicate in the presence of 11.1 mM succinate with substrate-activated mitochondrial preparations. Activation was carried out immediately prior to the assay by preincubating thawed mitochondria under N_2 for 15 min with 33.3 mM succinate at 30°C. For dilution, activation, and assay, buffer I (Materials and Methods) was used. The level of significance (P) is based on five preparations ($n = 5$) in both acclimation groups. Succinate oxidate system activity is expressed as ngatom O_2/mg protein per min. n.s., not significant.

Acclimation temperature	Succinate oxidase system activity		Q_{10}
	10°C	30°C	
10°C ($n = 5$)	128 ± 19	280 ± 42	1.48 ± 0.02
30°C ($n = 5$)	101 ± 6	219 ± 13	1.47 ± 0.01
P	<0.02	<0.02	n.s.

30°C. The reaction rates of the mitochondria from cold-acclimated carp exceed those of mitochondria from warm-acclimated carp by approx. 40%. Cold acclimation thus causes a partial compensation of the acute effect of decreasing temperature on the activity of cytochrome *c* oxidase. As indicated by the Q_{10} values of approx. 1.45 calculated for the 10 and 30°C activities, there is a remarkably weak dependence on temperature of cytochrome *c* oxidase activity which does not differ for either acclimation temperature of carp.

Succinate oxidase system. The affinity for succinate of the succinate oxidase system of red muscle mitochondria from carp has been reported to be independent of acclimation temperature and was characterized by K_m values near 0.40 mM [13]. Therefore, specific activities of the substrate-activated oxidase system obtained at 11.1 mM succinate practically represent maximum rates. Similar to the V values of cytochrome *c* oxidase, the data in Table II show that cold acclimation of carp causes a significant increase in maximum rates of the mitochondrial succinate oxidase system (cold exceeding warm by approx. 30%). Again Q_{10} values indicate an extremely weak dependence on temperature which does not change with acclimation temperature.

Cytochrome spectra

The specific activities of both oxidase systems of red muscle mitochondria studied here are enhanced during cold acclimation. This may be due to increased amounts of enzyme per mg mitochondrial protein or may reflect functional modulations of the oxidase systems remaining unchanged in concentration. For cytochrome *c* oxidase this question is open to direct experiment, since measurements of cytochrome aa_3 and protein provide the data necessary to calculate the enzyme concentration.

Fig. 2 shows two typcical reduced-minus-oxidized difference spectra for mitochondrial preparations from cold- and warm-acclimated carp (upper curves), the corresponding oxidized-minus-oxidized difference spectra are included (lower traces). The upper curves exhibit the expected α-peaks for cytochromes aa_3, b and $c + c_1$ at 605, 562 and 551 nm, respectively. Con-

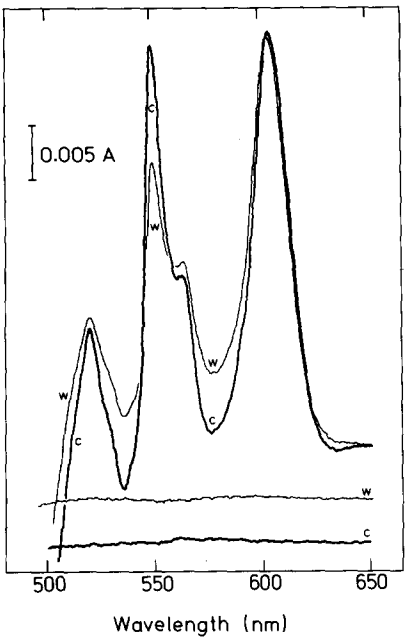

Fig. 2. Difference spectra of mitochondria from cold- and warm-acclimated carp. Cold-acclimated, 10°C (c); 1.69 mg protein/ml. Warm-acclimated, 30°C (w); 1.62 mg protein/ml. Upper curves: $Na_2S_2O_4$-reduced sample minus oxidized reference. Lower traces: oxidized sample minus oxidized reference (base line); the lower traces are vertically displaced.

sidering the mitochondrial protein concentration used in the experiments shown in Fig. 2, it would appear that there is no difference in the concentration of the terminal oxidase with respect to acclimation temperature. The reduced peak height at 551 nm and the less prominent troughs at about 535 and 575 nm in curve w, however, indicate a lower content of cytochrome $c + c_1$ in the mitochondria isolated from the warm-acclimated carp. This may be due to a significant extraction of cytochrome *c* from the 30°C-acclimated mitochondria during the isolation in the presence of KCl: warm acclimation of carp reduces mitochondrial phosphatidylethanolamine and thus decreases

TABLE III

THE CYTOCHROMES IN RED MUSCLE MITOCHONDRIA OF CARP ACCLIMATED TO COLD (10°C) AND WARM (30°C) TEMPERATURES

Data indicate mean ± S.D. of cytochrome concentrations determined from five preparations ($n = 5$) of both acclimation groups. Details are given in Materials and Methods. n.s., not significant.

Acclimation temperature	Cytochrome concentrations (nmol cytochrome/mg protein)		
	aa_3	b	$c + c_1$
10°C ($n = 5$)	0.85 ± 0.07	0.30 ± 0.05	0.87 ± 0.14
30°C ($n = 5$)	0.88 ± 0.07	0.25 ± 0.02	0.65 ± 0.03
P	n.s.	n.s.	<0.01

the number of slightly negative charges on the lipid bilayer at physiological pH [11,18].

The data for cytochrome aa_3 in Table III unambiguously show that the concentrations of the oxidase do not differ in mitochondria from warm- and cold-acclimated carp. Therefore, it is concluded that the observed differences in specific activity of cytochrome *c* oxidase (Table I) reflect changes in the functional capability of the enzyme within the membranes.

Discussion

The temperature dependence of the activities of mitochondrial succinate oxidation and cytochrome c oxidase

During cold acclimation of carp, the maximum activities of both mitochondrial membrane-bound enzymes examined in this study are increased. Evidence for lipid modulation of succinic dehydrogenase activity during thermal acclimation was presented by Hazel [10]. Correspondingly, in this study, the enhanced activity of the succinate oxidase system may reflect viscotropic regulation of the enzyme(s) caused by the increased unsaturation of mitochondrial phosphatidylcholine in red muscle of cold-acclimated carp [11]. Changes in the amount of enzyme(s) per mg mitochondrial protein, however, cannot be excluded. The situation for cytochrome *c* oxidase is discussed later.

Considerably low Q_{10} values were obtained for the activities of cytochrome *c* oxidase and the succinate oxidase system. Similarly, Arrhenius plots for succinate oxidation in liver mitochondria from eel [12] and carp [13] are characterized by E_a values approximately half of those reported for the mammalian enzyme system (Ref. 13 and references cited therein). Correspondingly, cytochrome *c* oxidase of the poikilothermic carp is less temperature sensitive as compared with the enzyme of a homeotherm: a Q_{10} value of 2.01 between 20 and 30°C has been reported for mammalian heart muscle mitochondria [19]. A comparison of the phospholipid unsaturation of the 'anchor' membranes suggests that again viscotropic regulation may cause these effects. The unsaturation index of phosphatidylcholine from the inner mitochondrial membranes of liver is 201 ± 12 for carp acclimated to 10°C (Pietschmann, R. and Wodtke, E., unpublished data) but only 124 for rat (calculated from Ref. 20). In the case of red muscle mitochondria, total lipid extracts exhibit unsaturation indices of 226 ± 10 for carp acclimated to 10°C and of 213 ± 10 for carp acclimated to 32°C [11], but only of 132 for porcine heart mitochondria (calculated from Ref. 21).

Apparently, there is an inverse correlation between membrane lipid unsaturation and the observed E_A and/or Q_{10} values of the membrane-bound enzymes. The definite causality, however, remains unclear (see review by Sandermann [22]).

Cytochrome concentrations in red muscle mitochondria

The cytochrome concentrations in mitochondria of carp red muscle are approx. 3-fold higher as compared with mitochondria from liver and gill of the goldfish [9] and with liver mitochondria of various fish species [6]. The value

for cytochrome aa_3 found in this study slightly exceeds the mean reported for fish heart mitochondria by Richardson et al. [23]. No interspecies difference in the cytochrome contents of fish liver mitochondria has been observed [6]. In a recent paper, considerable variation in the cytochrome concentrations of heart Keilin-Hartree particles from different fish was attributed to possible coprecipitation of non-mitochondrial protein [24]. A comparison of red muscle mitochondria of the carp with mitochondria from different muscles of higher vertebrates shows that cytochrome concentrations are similar in mitochondria from beef heart [25] and pigeon breast muscle [26] but lower for rat leg muscle [26]. Though no data are available for white skeletal muscle of fish, the above comparison suggests that the close relationship between mitochondrial cytochrome concentrations and the aerobic/anaerobic type of muscle function is not limited to homoiotherms but may be valid, at least within vertebrates, irrespective of body temperature. Correspondingly, the acclimation temperature of carp does not affect the stoichiometry of cytochromes and mitochondrial protein. This is in agreement with reported earlier results for goldfish [9].

The affinity of cytochrome c oxidase for cytochrome c

Linear Eadie-Hofstee plots were obtained for the membrane-bound cytochrome *c* oxidase in mitochondria from cold- and warm-acclimated carp. The corresponding K_m values for cytochrome *c* are about 3.3 μM. (30°C, pH 7.5) and thus within the range reported for rat liver mitochondria [27] and are in agreement with 3.1 μM (25°C, pH 7.4) as measured with carp liver mitochondria (Pietschmann, R. and Wodtke, E., unpublished data). In these experiments, buffers of comparable ionic strenghth were used and cytochrome *c* concentrations were 0.75 μM and higher.

According to Ferguson-Miller et al. [28], biphasic Eadie-Hofstee plots have been obtained by Wilson et al. [24] for Keilin-Hartree particles of fish heart. In this case, assays were performed at lower ionic strength, and lower cytochrome *c* concentrations were included. The K_m values (29°C, pH 7.4) calculated for the low-affinity site range from 0.7 to 2 μM cytochrome *c* for different fish species.

Apparently, the variation in K_m values for the low-affinity site obtained with various fish and mammalian species is rather small. Therefore, it is not surprising that the acclimation temperature of carp does not affect the K_m value for cytochrome *c* of cytochrome *c* oxidase in red muscle mitochondria.

Maximum activity of cytochrome c oxidase

The acclimation temperature of carp does not affect the amount of cytochrome *c* oxidase per mg mitochondrial protein nor does it affect the substrate binding properties of the enzyme as judged from the K_m values/saturation kinetics for cytochrome *c*. Thus it would appear reasonable to assume that there is no difference in quantity and structural quality of the oxidase proteins with respect to acclimation temperature.

In contrast, an acclimation temperature-induced functional difference is observed in the maximum activity of the enzyme. The molar activities at 30°C in nmol ferrocytochrome *c*/nmol heme *a* per s are 117.6 ± 17.8 for the

enzyme intercalated in the cold-acclimated membrane, but 85.6 ± 17.2 in the case of the warm-acclimated one.

The molar activities reported for cytochrome *c* oxidase depend on assay conditions, they generally cover the range from less than 100 to 300 mol cytochrome *c* per mol heme *a* [29,30]. Including phosphate in the assay medium has been shown to increase the activity of the oxidase by a factor of 3 [9], 2 [31], or 1.5 [27]. In gill mitochondria of cold (warm) acclimated goldfish cytochrome *c* oxidase exhibits molar activities of 170 (123) at 20°C in the presence of 77 mM phosphate [9]. Considering the lower assay temperature and the 3-fold activity rise mediated by phosphate, the data of Caldwell [9] come close to those obtained in this study at 30°C without phosphate.

It is known that cytochrome *c* oxidase requires the presence of phospholipids (or non-ionic detergent) for activity [30]. An essential role of cardiolipin has been postulated, since this phospholipid has been reported to be tightly associated with the oxidase [32—35] and, upon readdition to lipid-depleted inner mitochondrial membranes, most effectively restores the original protein conformation [26].

Capaldi and coworkers [34,35] have shown that approx. 10 mol of cardiolipin per mol of ox heart cytochrome *c* oxidase are resistent to detergent exchange even in the presence of a 100-fold molar excess of dimyristoyl phosphatidylcholine. As calculated in the Appendix, at physiological temperatures, these tightly bound cardiolipin molecules might be of sufficient number to create a dynamic shell separating the hydrophobic intrinsic parts of the enzyme from the adjacent membrane phospholipids. Accepting this 'annular' model, the higher activity of the cardiolipin-oxidase complex in the presence of dioleoyl phosphatidylcholine as compared with dipalmitoyl phosphatidylcholine [35] would then suggest that the bilayer fluidity and not the annular fluidity determines the activity of cytochrome *c* oxidase.

Indeed, this interpretation is in harmony with the effects of acclimation temperature on carp red muscle mitochondria. Cold acclimation elevates the molar activity of cytochrome *c* oxidase (this paper) and increases the unsaturation of phosphatidylcholine, but decreases the unsaturation of cardiolipin as reported and discussed in the preceding paper [11].

It is concluded that viscotropic regulation of the activity of cytochrome *c* oxidase in red muscle mitochondria occurs during thermal acclimation of the carp. This might be mediated by the control of motional freedom of the oxidase lipoprotein within the mitochondrial bilayer and/or secondly by the control of freedom for two-dimensional diffusion of cytochrome *c* on the membrane surface.

Appendix

On the possible association of cardiolipin with cytochrome c oxidase in the inner mitochondrial membrane

From the examination of membranous cytochrome oxidase by ESR spectroscopy, Jost et al. [37] obtained evidence for a lipid layer firmly bound to the hydrophobic surface of cytochrome *c* oxidase. Under appropriate conditions, the enzyme from beef heart can be isolated with 10 mol of cardiolipin plus

2—3 mol of neutral phospholipids tightly bound to 1 mol of enzyme [34,35]. Are these lipids sufficient in number to constitute a shell around the oxidase?

(a) From the diameter of the enzyme protein, approx. 55 Å [38], its circumference is calculated to be about 173 Å.

(b) From the area of 85 $Å^2$ per molecule of 1,2-dilinoleoyl phosphatidylcholine in a monolayer at 20 dyne/cm and 22°C [39], the dynamic diameter of one *cis,cis*-diunsaturated acyl chain (mainly constituting mammalian cardiolipin) is calculated to be $\sqrt{85/2} = 6.5$ Å at 22°C. (The cross-sectional area of one chain is treated as a square, the lateral length of which is taken as the chain diameter.)

(c) From pressure-area curves at various temperatures [40], it can be calculated that elevating the temperature of a monolayer from 21 to 35°C increases its area approx. 1.4-fold. The linear extension is thus increased by $\sqrt{1.4} = 1.18$-fold.

(d) From b and c, the dynamic diameter of one *cis,cis*-diunsaturated acyl chain is calculated to be approx. 7.7 Å at the physiological temperature for beef heart function.

(e) From a and d, it follows that 173/7.7 acyl chains or $173/7.7 \times 4 = 5.62$ molecules of cardiolipin would be sufficient to create an annulus in one leaflet of the bilayer.

(f) Since the oxidase spans the membrane, complete shielding requires 11.24 molecules of cardiolipin or 10 molecules of cardiolipin plus $1.24 \times 2 = 2.5$ molecules of neutral phospholipids (e.g., phosphatidylcholine or phosphatidylethanolamine).

From the calculations a—f, it would appear possible that the tightly bound, detergent-exchange-resistent lipids of the enzyme preparation of Capaldi and coworkers [34,35] are sufficient in number to constitute a dynamic shell separating the hydrophobic intrinsic parts of cytochrome *c* oxidase from the adjacent membrane phospholipids under physiological conditions. Less cardiolipin and/or cone-shaped cardiolipin [41] might just fill the notches and grooves in the hydrophobic surface of the oxidase protein thus creating a smooth-surface lipoprotein.

The results of this calculation support the idea that the negatively charged cardiolipin constitutes an annulus around the cytochrome *c* oxidase protein in vivo. This is not in contrast to the study of Seelig and Seelig [42] in which lipid-free cytochrome *c* oxidase was reconstituted with zwitterionic, i.e., noncharged, phosphatidylcholine and no evidence for a special type of boundary lipid was found.

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