REVERSIBILITY OF THE INHIBITION OF CYTOCHROME *c* OXIDASE BY RETICULOCYTE LIPOXYGENASE

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1. Introduction

During the maturation of reticulocytes the mitochondria are lyzed and inactivated by a cell-specific lipoxygenase (LOX) [1]. In addition to oxygenation of mitochondrial phospholipids the LOX action gives rise to an inhibition of the respiratory chain at three sites. Two inhibitory sites are located between the Fe–S centers of complex I and complex II and ubiquinone [2]. The third inhibitory site located at the cytochrome *c* oxidase differs from the others with respect to the kinetics, dose-dependence and the extent of the inhibition [3,4]. The present paper deals with the mechanism of the inhibitory action of reticulocyte LOX on particulate cytochrome *c* oxidase. As reported elsewhere, even extensive LOX-treatment of submitochondrial particles (SMP) does not affect the amount and the spectral properties of the redox-active components of the cytochrome *c* oxidase [1,2], which rules out the possibility of co-oxidative destruction of these components. In this paper evidence will be adduced that LOX inhibits cytochrome *c* oxidase by chemical modification of the membrane phospholipids which are essential for its activity.

2. Materials and methods

Beef heart mitochondria, SMP and Keilin-Hartree heart muscle preparation were prepared by standard methods [5,6]. LOX was purified from rabbit reticulocytes [1] and from soybeans [7]. The extraction of phospholipids from untreated and LOX-treated mitochondria was performed both with acetone–water 95:5 [8] and with chloroform–methanol–0.1 M KCl 10:5:3 [9] under nitrogen purified by both alkaline pyrogallol and BTS catalyzer (Fluka AG, Switzerland). For the reactivation experiments 40 mg phospholipids were suspended in 4 ml 0.02 M Tris–acetate containing 1 mM EDTA (pH 8.0) and sonified 3 times for 3 min with cooling under nitrogen (tip-type sonifier, Branson Sonic Power Co., USA, microtip); the suspension was centrifuged at 15 000 rev./min for 20 min (Sorvall SS 3, Sorvall-DuPont, USA). Phospholipids from soybeans (Asolectin, commercial grade, type II-S, Sigma, USA) and Tween 80 (Atlas-Powder Co. USA) were used without further purification.

Thin-layer chromatography (TLC) of the phospholipids was performed according to [11]. Cytochrome *c* oxidase activity was assayed both spectrophotometrically using ferrocytochrome *c* (Serva, FRG) [10] and polarographically using ascorbate/TMPD [12] in 0.1 M potassium phosphate, pH 7.4 at 37°C. Protein concentrations were determined by the Biuret method [13].

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

3. Results

Table 1 shows the reversal of the LOX-mediated inhibition of cytochrome *c* oxidase by subsequent treatment with soybean phospholipids or Tween 80. The phospholipids restored the activity completely, whereas Tween 80 produced only partial reactivation. These results are in good agreement with those of Vik and Capaldi who used cytochrome *c* oxidase depleted of endogenous lipid by detergent exchange [14]. Similar results (not shown) were obtained with SMP and with Keilin-Hartree heart muscle preparations. With LOX-treated SMP soybean phospholipid caused even

Table 1
Reversal of inhibition of cytochrome *c* oxidase in mitochondria by soybean phospholipid and Tween 80

LOX-treatment of mitochondria	Addition for reactivation	Cytochrome *c* oxidase activity (ΔA_{550}/min)
–	–	0.430
+	–	0.092
+	Phospholipid (5 mg/ml)	0.445
+	1% Tween 80	0.280
+	3% Tween 80	0.300

The mitochondria (2.6 mg protein) were preincubated twice with 5 mg LOX each at 37°C for 30 min (final vol. 0.3 ml in 0.25 M sucrose/10.6 mM K_2HPO_4). 5 μl of the diluted (1:30) preincubation sample were added to the reactivation sample (200 μl) containing soybean phospholipid or Tween 80 in the final concentration as indicated. After incubation at 30°C for 10 min the cytochrome *c* oxidase activity was measured at 37°C spectrophotometrically (mean values of triplicate assay)

Table 2
Reversal of inhibition of cytochrome *c* oxidase in SMP by phospholipids

LOX-treatment of SMP	Addition for reactivation	Cytochrome *c* oxidase activity (ΔA_{550}/min)
–	–	0.215
+	–	0.125
+	Soy-PL	0.335
+	MPL	0.250
+	MPL (LOX)	0.100

SMP (0.2 mg protein) were preincubated twice with 2.5 mg LOX each at 37°C for 30 min (final vol. 0.2 ml in 0.25 M sucrose/10.6 mM K_2HPO_4). 10 μl of the preincubation sample were subjected to reactivation by 1 mg phospholipid as indicated in table 1

Abbreviations: Soy-PL, soybean phospholipids; MPL, mitochondrial phospholipids; MPL (LOX), phospholipids of LOX-treated mitochondria

additional activation as compared with the control of untreated SMP (table 2). This additional activation was observed, however, only when the activity was measured with external ferrocytochrome *c*, but not with ascorbate/TMPD as electron donor (table 3). This may indicate that LOX treatment with subsequent phospholipid treatment improves the accessibility of the SMP to external ferrocytochrome *c*, which is hindered in 'inside out' vesicles normally present in SMP preparations. The cytochrome *c* oxidase activity of LOX-treated SMP was also restored with native mitochondrial phospholipids. In contrast, a phospholipid extract of LOX-treated mitochondria failed to restore the activity (table 2). TLC of the phospholipids of LOX-treated mitochondria revealed a strong decrease in the share of phosphatidyl ethanolamine which is known to have a high content of arachidonic acid [15]; on the other hand, the content of strongly polar non-migrating compounds was enhanced. The phospholipid extract did not gave a positive reaction with thiobarbituric acid indicating that the reaction of the LOX did not stop at hydroperoxides but led

Table 3
Reactivation by soybean phospholipid of cytochrome *c* oxidase of SMP as measured by two different assays

LOX-treatment of SMP	Addition for reactivation		Cytochrome *c* oxidase activity on	
	1 mM SHAM	Phospholipid	Asc./TMPD ΔO_2 [mmol/min]	Ferrocyt. *c* ΔA_{550}/min
–	–	–		0.158
+	–	–		0.090
–	+	–	72	0.175
+	+	–	30	0.080
–	+	+	50	0.100
+	+	+	49	0.275

SMP (0.22 mg protein) were preincubated with 2.5 mg LOX at 37°C for 30 min under occasional shaking (final vol. 200 μl in 0.25 M sucrose/10.6 mM K_2HPO_4) then SHAM was added and incubated for another 5 min. Reactivation by 100 mg soybean phospholipid/mg SMP protein was performed as indicated in table 1. The reactivated SMP were washed in sucrose medium and resuspended in 200 μl. 50 or 10 μl were added to the asc./TMPD or ferrocytochrome *c* system, respectively

further to the formation of non-peroxidic products.

A reactivation of the cytochrome *c* oxidase by phospholipids in LOX-treated mitochondria was also observed after extraction with acetone–water 95:5 which is known to remove 70–75% of the total mitochondrial phospholipid [16]. Reconstitution with native phospholipids after acetone extraction led to a comparable reactivation of LOX-treated and untreated mitochondria. The total lipid extract of LOX-treated mitochondria after addition of fresh LOX exhibited only 7% of the oxygen uptake as compared with that of the lipid extract of untreated mitochondria. On the other hand, the acetone-treated mitochondria showed only a negligible oxygen uptake after addition of LOX in contrast to non-extracted mitochondria, although the acetone-treated mitochondria retained ~20% of their original cytochome *c* oxidase activity. This suggests that acetone extraction removes that portion of mitochondrial phospholipids which is also accessible to LOX. In contrast to the cytochrome *c* oxidase activity the succinate–cytochrome *c* oxidoreductase activity of LOX-treated mitochondria could not be restored by phospholipids plus ubiquinone-50 either with or without acetone-treatment. The action of LOX on the succinate–ubiquinone oxidoreductase system is obviously not restricted to the phospholipid moiety, but leads secondarily to irreversible damage of protein components.

The reversal by phospholipids of the LOX-mediated inhibition of the cytochrome *c* oxidase in SMP required an ~250-fold excess of added phospholipids as referred to the endogenous phospholipid content. Only partial reactivation was observed with half the amount.

Table 3 shows that the site of both inhibition by LOX and the subsequent reactivation by phospholipids is the cytochrome *c* oxidase complex per se rather than its interaction with external ferrocytochrome *c*, since identical results were obtained with the ascorbate/TMPD oxidase system which works without external cytochrome *c*. In this experiment an addition of the LOX inhibitor salicylhydroxamic acid (SHAM) after the LOX-treatment was necessary in order to avoid an interference by LOX-mediated oxygen uptake upon the addition of phospholipid in the polarographic assay. An addition of SHAM before LOX treatment prevented totally the inhibition of cytochrome *c* oxidase as measured polarographically (not shown). The partial inhibition of the untreated SMP by phospholipid may be due to their content of free fatty acids (detected by TLC) which are able to inhibit the cytochrome *c* oxidase [17].

In contrast to reticulocyte LOX a comparable amount of soybean LOX-1 as referred to the activity on linoleate as substrate failed to produce both oxygen uptake with mitochondria or inhibition of cytochrome *c* oxidase.

4. Discussion

This report demonstrates the reversibility of the inhibition of cytochrome *c* oxidase by LOX and rules out the possibility of a secondary damage of polypeptide components of the cytochrome *c* oxidase complex. The mechanism of the reversal by phospholipids may be explained on the basis of the recent findings by Schneider et al., who have demonstrated a fusion of soybean phospholipid liposomes with mitochondrial inner membranes [18]. The high excess of phospholipid necessary for reactivation in our experiments may be due to the unfavorable conditions for such a fusion (e.g., pH 7.4 instead of 6.5). The added phospholipids which are incorporated in the LOX-treated inner mitochondrial membranes displace presumably the phospholipids modified by LOX and thus restore the lipid environment needed for the activity of the cytochrome *c* oxidase system. The reverse action, i.e., a displacement of phospholipids by the inactive modified ones is suggested in experiments where they were added (not shown). The attack of LOX on the fatty acid moiety of phospholipids has two consequences:

(i) A lowering of the hydrophobicity owing to the introduction of hydroperoxy or (secondarily) hydroxy- and other hydrophilic-groups;

(ii) A decrease in the membrane fluidity.

The first effect weakens the hydrophobic interactions with catalytic proteins which are essential for their activity. As far as the fluidity of the phospholipids is concerned, Vik and Capaldi have shown in their reconstitution experiments, that substitution of saturated phospholipids for the dioleyl derivatives or natural phospholipids reduces the cytochrome *c* oxidase activity by at least 50%, whereas the polar group is of minor importance.

The reticulocyte LOX has turned out to be an interesting tool for the study of phospholipid-dependent enzymes.

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