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ON THE SITE OF ACTION OF THE INHIBITION OF THE MITOCHONDRIAL RESPIRATORY CHAIN BY LIPOXYGENASE

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Treatment of beef-heart submitochondrial particles with reticulocyte lipoxygenase gives rise to a strong irreversible inhibition of the NADH and succinate oxidase activities. This is not accompanied by any loss of the Fe-S clusters of the respiratory chain as determined by EPR spectroscopy. The inhibitory blockage is located between both the NADH and succinate dehydrogenases and Q-10. The inhibitory action of treatment with lipoxygenase also takes place in the absence of Q-10. The Fe-S clusters of the mitochondrial outer membrane are destroyed by lipoxygenase treatment, without any effect on the rotenone-insensitive NADH : cytochrome *c* oxidoreductase activity. It is concluded that these clusters are not involved in this enzyme.

Introduction

A respiration-inhibitory protein in rabbit reticulocytes has been detected by Rapoport et al. in 1955 [1,2]. Later a lipoxygenase which caused a strong lysis of mitochondria in vitro was found in these cells [3,4]. Recently, it has been proven that both protein factors are identical [5]. Now the question arises as to the mechanism of action of the respiratory inhibition produced by lipoxygenase. In a former study the Fe-S regions of NADH : Q oxidoreductase have been suggested as the sites of action [6]. This suggestion seemed to have been supported by the observation of a partial loss of acid-labile sulphur after lipoxygenase treatment of submitochondrial particles [7]. In this paper, the behaviour of the Fe-S clusters was studied by EPR spectroscopy. The results exclude the possibility that the Fe-S clusters of the respiratory chain are points of attack for lipoxygenase and allow a more precise location of the site of action.

Materials and Methods

Submitochondrial particles from beef heart were prepared according to Crane et al. [8] with the exception that disintegration of the mitochondria was performed by sonification (Branson sonified, three times at 1 min each at half-maximal output, 0–15°C). The particles were stored in liquid N_2 until use. Q-depleted particles were prepared by pentane extraction [9]. The replenishment was performed [10] using a solution of Q-10 in pentane (15 nmol/mg protein).

Lipoxygenase from rabbit reticulocytes was prepared as described earlier [5]. Owing to its higher stability, the dialyzed $(NH_4)_2SO_4$ precipitate was used rather than the pure enzyme. Submitochondrial particles were incubated with an excess of lipoxygenase (as referred to the respiratory inhibition obtained) for 15 min at 37°C if not stated otherwise. The amount of lipoxygenase necessary to

Abbreviations: Q, ubiquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCIP, dichlorophenolindophenol.

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achieve an inhibition of the NADH oxidase activity of 50% was found to be about 0.4 nmol/mg protein of submitochondrial particles as judged from experiments with highly purified lipoxygenase. For the EPR studies, lipoxygenase treatments was carried out with dense suspensions of particles (46 or 58 mg/ml). Samples were withdrawn for activity measurement and when the inhibitory action had proceeded far enough, the reaction was stopped by chilling the suspension in an ice/water mixture. The suspension was either used directly or the particles were washed with a 40-fold volume of buffered sucrose and resuspended in the original volume.

EPR spectra were recorded on a Varian E-9 EPR spectrometer. Analysis of the spectra was performed as described earlier [11]. The activity measurements of the partial systems of the respiratory chain were carried out spectrophotometrically or with an oxygen electrode [12] at 37°C. Linoleate lipoxygenase activity was measured by monitoring the conjugated diene formation at 234 nm at 15°C in the assay mixture reported earlier [5]. In some experiments 5 mM Q-10 or an equimolar mixture of Q-10 and linoleate was substituted for linoleate.

Results

The effect of lipoxygenase treatment on various enzymatic systems of submitochondrial particles is shown in Table I. Strong inhibition appears in all systems that involve reduction of Q by substrate. The NADH dehydrogenase activity measured with $K_3Fe(CN)_6$ as acceptor is not affected. The partial inhibition of the succinate-phenazine methosulphate oxidoreductase as well as of the TMPD bypass is consistent with the almost complete absence of a succinate: Q-1 oxidoreductase activity [13]. Lipoxygenase treatment probably abolishes communication between the succinate dehydrogenase and ubiquinone. This conclusion is based on the fact that the residual succinate-phenazine methosulphate oxidoreductase activity is inhibited by 2-thenoyltrifluoroacetone competitively with respect to phenazine methosulphate (Fig. 1). This is also the case in preparations of succinate : Q oxidoreductase lacking Q-10 [13,14].

In agreement with an earlier finding [15], the activity of cytochrome *c* oxidase with ferrocytochrome *c* or ascorbate/TMPD as electron donors is also partly inhibited by lipoxygenase. The rotenone-insensitive NADH : cytochrome *c* oxidoreductase activity which is located in the mitochondrial outer membrane [16] is resistant to lipoxygenase treatment.

In view of the expectation that Fe-S clusters in both NADH dehydrogenase and succinate dehydrogenase would be the target of lipoxygenase, extensive EPR studies were carried out. Optimal EPR conditions for each of the individual Fe-S clusters, as specified in previous studies [17–19,11], were used. Under each condition, submitochondrial particles treated with lipoxygenase were compared with untreated particles from the same batch. The lipoxygenase treatment had, however, no effect at all, either on the line shape or on the intensity of the signals of the individual Fe-S clusters 1a, 1b, 2, 3 and 4 [17–19] of NADH dehydrogenase. The same results were obtained in experiments in which two extra lipoxygenase additions were made to a standard incubation mixture after 15 and 30 min, respectively, and where the total incubation time was

TABLE I

EFFECT OF LIPOXYGENASE TREATMENT ON VARIOUS ENZYMATIC ACTIVITIES OF BEEF-HEART SUBMITOCHONDRIAL PARTICLES

Some of the values were taken from Ref. 6.

Oxidoreductase reaction tested	Residual activity (%)
NADH oxidase	7
NADH: ferricytochrome *c*, rotenone-sensitive	10
NADH: ferricytochrome *c*, rotenone-insensitive	100
NADH: Q-1	14
NADH: $K_3Fe(CN)_6$	88
NADH: menadione	87
Succinate: O_2	9
Succinate: ferricytochrome *c*	12
Succinate: Q-1: DCIP	15
Succinate: phenazine methosulphate: DCIP	40
Succinate: TMPD: O_2 (with antimycin A)	38
QH_2-6: ferricytochrome *c*	100
Ferrocytochrome *c*: O_2	60
Ascorbate: TMPD: O_2	58

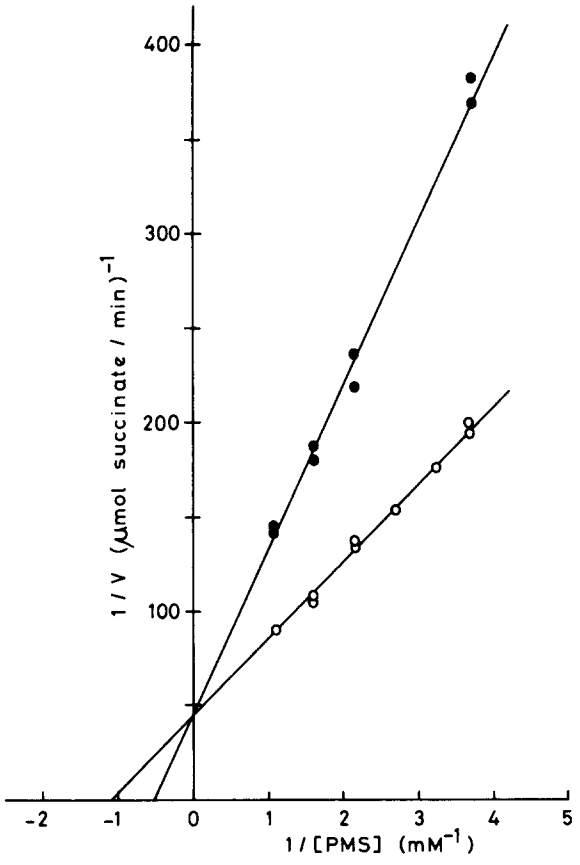
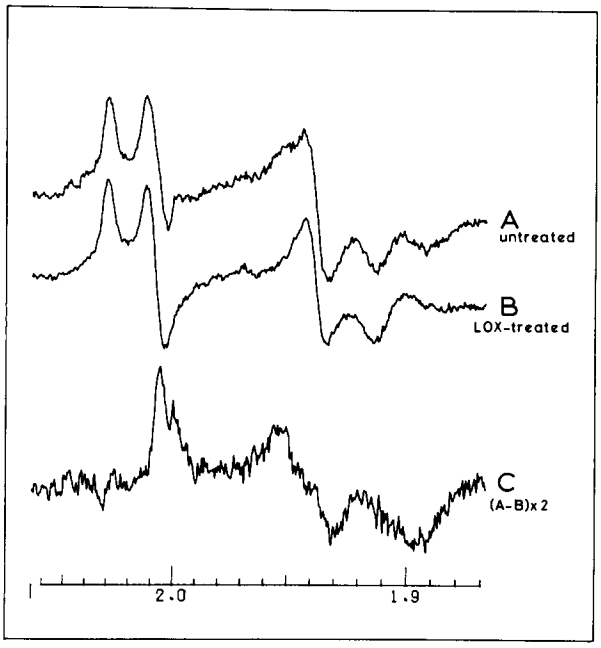


Fig. 1. Inhibition by 2-thenoyltrifluoroacetone of succinate : phenazine methosulphate oxidoreductase of lipoxygenase-treated submitochondrial particles. The assay mixture (37°C) contained submitochondrial particles (24 µg of protein/ml) previously treated with an excess of reticulocyte lipoxygenase for 15 min at 37°C, 100 mM potassium phosphate buffer (pH 7.5), 45 µM DCIP, 20 mM succinate and 2 mM KCN. No inhibitor (○), with 0.75 mM 2-thenoyltrifluoroacetone present (●). The particles were activated by preincubation for 5 min at 37°C with 20 mM succinate. The reaction was started with phenazine methosulphate 2 min after addition of 2-thenoyltrifluoroacetone. Untreated particles showed an inhibition of 48% with 0.75 mM 2-thenoyltrifluoroacetone and 55% after the lipoxygenase treatment, both measured at a phenazine methosulphate concentration of 0.96 mM. PMS, phenazine methosulphate.

prolonged up to 45 min, reaching an inhibition of the NADH oxidase activity of 98%. A single addition of lipoxygenase caused an inhibition of not higher than about 90% owing to the suicidal nature of the reaction of the enzyme with its substrates [5]. It was observed that when lipoxygenase-treated submitochondrial particles were mixed with NADH for 15 s at 4°C, all EPR signals of NADH dehydrogenase were fully detectable, whereas the Fe-S clusters of succinate dehydrogenase [11] and QH_2 : cytochrome *c* oxidoreductase [20] as well as the EPR-detectable Cu of cytochrome *c* oxidase remained oxidized. In untreated particles, all components are reduced under these conditions as the dense suspension runs rapidly anaerobic. Therefore, the blockage by lipoxygenase of the respiratory chain seems to be in the reduction of ubiquinone.

Similar results were obtained with the Fe-S clusters of succinate dehydrogenase. Here again both the binuclear and the tetranuclear clusters [11] remained unchanged in amount and line shape upon a single lipoxygenase treatment for 25 min leading to an inhibition of more than 90% of the succinate : Q-1 oxidoreductase activity. However, upon prolonged incubation with two subsequent additions of lipoxygenase to the incubation mixture, a considerable amount of the [4Fe-4S] cluster was destroyed. Concomitantly, a corresponding fraction of cluster 1 lost its ability to become reduced by succinate. These changes paralleled a further loss of succinate-phenazine methosulphate oxidoreductase activity. The destruction of particulate succinate dehydrogenase cannot, however, be the primary point of attack by lipoxygenase on the succinate oxidase activity, since the respiratory inhibition starts much earlier.

The submitochondrial particles used in our experiments represent a mixture of inner and outer membranes [21]. The outer membranes contain two Fe-S clusters in a 2 : 1 ratio [22]. We could observe a specific and complete loss of these clusters in submitochondrial particles upon treatment with lipoxygenase. This is demonstrated in Fig. 2. The effect of lipoxygenase treatment can be observed in 49 K EPR spectra of particles reduced with dithionite. In this case, the spectrum is a composite of the spectra of cluster 1 of succinate dehydrogenase, clusters 1 and 2 of QH_2 : cytochrome *c* oxidoreductase and the outer-membrane Fe-S clusters. The clusters 1a/1b of NADH dehydrogenase are not reduced by a short incubation with dithionite. The effect of lipoxygenase treatment is, however, only obvious in a difference spectrum (Fig. 2, trace C). As the spin-lattice relaxation of the outer-membrane Fe-S



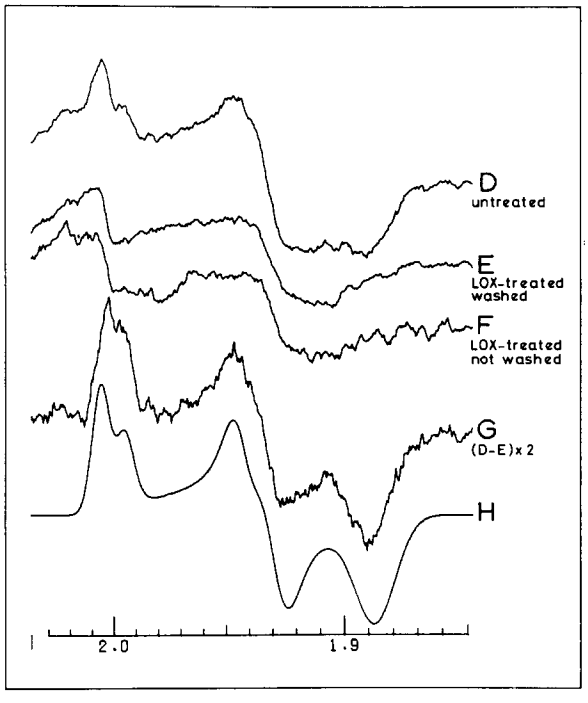


Fig. 2. EPR spectra of submitochondrial particles before and after a treatment with lipoxygenase (LOX). Top panel: (A) untreated particles reduced with a few grains of solid $Na_2S_2O_4$ for 1 min at 0°C. (B) Particles, treated with lipoxygenase until the NADH oxidase activity was inhibited by 97%. Reduction as in A. The protein concentrations in A and B were the same. (C) 2-times enlarged difference spectrum A–B. All spectra were converted to the same microwave frequency. EPR conditions: microwave frequency, 9334.6 MHz; temperature, 49 K; incident microwave power, 2.2 mW; modulation amplitude, 0.63 mT; scanning rate, 1 mT/min. The modulation frequency for this and the other EPR spectra was 100 kHz. The x-axis scale refers to g values. Bottom panel: (D) untreated particles, the same as for trace A, reduced with 8 mM NADH for 30 s at 0°C and subsequently with $Na_2S_2O_4$ for 1 min at 0°C. (E) Particles treated with lipoxygenase, the same as used for trace B, reduced as in D. (F) Particles treated with lipoxygenase and examined without washing. Reduction as in D. (G) 2-times enlarged difference spectrum D–E. (H) Simulation of the EPR spectrum of mitochondrial outer membranes under similar conditions. For parameters see Fig. 5 of Ref. 22. EPR conditions: microwave frequency, 9149.9 MHz; temperature, 133 K; incident microwave power, 123 mW; modulation amplitude, 0.63 mT; scanning rate, 2 mT/min.

clusters is much slower than of the clusters in the respiratory chain [23], the effect of lipoxygenase treatment is more clearly observed at higher EPR observation temepratures (Fig. 2, traces D–G). Here the signal mainly consists of those from the outer-membrane Fe-S clusters, whereas the other signals are greatly broadened by relaxation. The difference spectrum (Fig. 2, trace G) clearly shows the characteristics of the EPR signal displayed by outer membranes (trace H). If washing of the particles after treatment with lipoxygenase was omitted, the same EPR spectrum was observed (Fig. 2, traces E and F) suggesting that the outer-membrane Fe-S clusters are actually destroyed rather than solubilized by lipoxygenase. This conclusion is strengthened by the observations, also made earlier [7], that acid-labile sulphur is lost during the lipoxygenase treatment (Fig. 3).

EPR traces at 15 K of the g_z line at $g = 3$ of the signal of cytochrome *a* and spectra at 50 K of the accompanying Cu atom [25] revealed the total absence of any effect of lipoxygenase. The cytochrome *a* and the accompanying Cu atom are thus not targets for the inhibitory action of lipoxygenase on cytochrome *c* oxidase.

The EPR investigations rule out the possibility that one of the EPR detectable components of the respiratory chain is the primary site of the inhibitory action of lipoxygenase. Since EPR spectra of paramagnets of the respiratory chain are well documented in the literature [17–19,11, 25–28] and since we

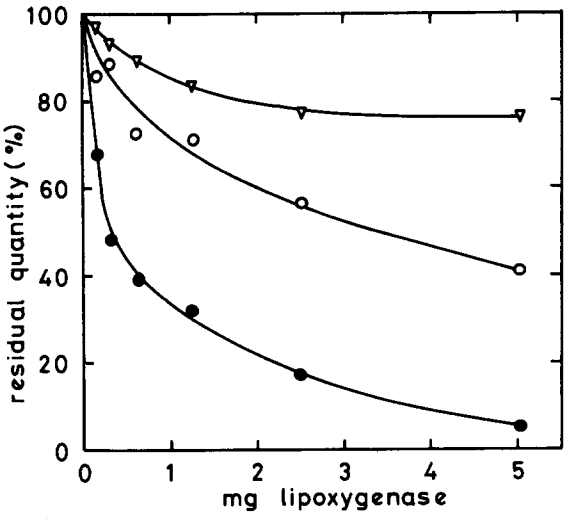


Fig. 3. Decrease in acid-labile sulphur content of submitochondrial particles upon treatment with lipoxygenase. Submitochondrial particles (2.7 mg of protein) were incubated for 15 min at 37°C with different amounts of lipoxygenase. The reaction was stopped by cooling in an ice-water bath. The acid-labile sulphur content (▽) [24] as well as the NADH oxidase (●) and succinate oxidase (○) activities were then determined.

could not observe any effect of a single lipoxygenase treatment on any of these EPR spectra of submitochondrial particles, the spectra are not included.

The lack of any effect of lipoxygenase treatment and the effects on the various activities listed in Table I sugggested the possibility that Q-10 is destroyed by lipoxygenase. Indeed, in a preliminary experiment we were not able to detect Q-10 in methanol/petroleum ether extracts [29] from lipoxygenase-treated particles in contrast to the controls. Although the fate of Q-10 upon lipoxygenase treatment requires a separate study and a loss of Q-10 alone would explain the respiratory inhibition, we have ample evidence that Q-10 is not the primary point of attack. One strong argument comes from experiments with Q-depleted submitochondrial particles (Table II). When the Q-depleted particles were treated with lipoxygenase and subsequently replenished with Q-10, neither NADH oxidase nor succinate oxidase activity was restored in contrast to particles not treated with lipoxygenase. This means that the action of lipoxygenase appeared even in the absence of endogenous Q-10. No conjugated diene [5], measured by absorbance at 234 nm [5], was formed by mixing Q-10 and lipoxygenase. Nor was there any effect of Q-10 on the rate of diene formation from linoleate. All these observations lead us to the conclusion that Q-10 cannot be the primary site of action of lipoxygenase, even though a co-oxidative destruction of Q-10 in normal submitochondrial particles cannot be excluded.

In contrast to normal particles, the Q-depleted particles showed partial damage of the NADH-ferricyanide activity upon lipoxygenase treatment. It is possible that Q protects a part of the respiratory chain from the action of lipoxygenase.

TABLE II

ACTION OF RETICULOCYTE LIPOXYGENASE ON Q-DEPLETED SUBMITOCHONDRIAL PARTICLES

Activities are given in μmol substrate/mg protein per min. LOX = lipoxygenase; SMP = submitochondrial particles; n.m. = not measured; PMS, phenazine methosulphate.

	NADH oxidase	NADH: ferricyanide oxidoreductase	Succinate oxidase	Succinate: PMS oxidoreducase
SMP	0.259	n.m.	0.215	1.07
Q-depleted SMP	0.010	n.m.	n.m.	n.m.
Q-replenished SMP	0.116	2.59	0.145	0.72
SMP (LOX-treated)	0.014	n.m.	n.m.	0.58
Q-depleted SMP, then LOX-treated and Q-replenished	0.005	1.65	0.018	0.26

Discussion

The EPR experiments described here and in former experiments using optical spectroscopy [5] show clearly that no prosthetic group in the respiratory chain is destroyed during moderate lipoxygenase treatment leading to a nearly total inhibition of NADH and succinate oxidase activities. Since haemoproteins such as cytochrome *c* (Schewe, T., Härtel, B., Hiebsch, C. and Rapoport, S.M., unpublished observations) and Fe-S proteins (in mitochondrial outer membranes, this study) are co-oxidatively destroyed in model systems containing reticulocyte lipoxygenase and one of its substrates, it is likely that the special assembly of the mitochondrial inner membrane affords protection against the lipoxygenase-mediated destruction of haem compounds and Fe-S clusters.

The primary target of the lipoxygenase action on the mitochondrial inner membrane must be the polyene fatty acid residues of the phospholipids that are known to be essential for the functionality of the respiratory chain. The action of the reticulocyte lipoxygenase on pure mitochondrial phospholipids was reported elsewhere [3]. By introducing the polar hydroperoxy group, the lipoxygenase attack renders the fatty acid residues more hydrophilic. This may weaken the hydrophobic lipid-protein interactions required for the enzymic activity of the electron-transfer system. From the EPR experiments, it is evident that the oxygenation of the mitochondrial inner membrane phospholipids does not severely affect the ability of the Fe-S clusters of the NADH dehydrogenase to be reduced by NADH, whereas the reduction of the subsequent electron carriers of the respiratory chain is strongly inhibited.

The inhibition of the succinate oxidase activity cannot be reversed by subsequent addition of native mitochondrial or other phospholipids plus Q-10, whether or not the peroxidized phospholipids are extracted from the particles by 90% acetone [30]. Thus, this type of respiratory inhibition cannot be explained solely as a consequence of the chemical modification of membrane phospholipids. It is conceivable that irreversible consecutive reactions of the lipohydroperoxides with protein moieties are responsible for the interruption of electron fluxes at the Q-reducing sites. There is now some evidence that not only the lipoxygenase activity per se but also the lipohydroperoxidase activity of this enzyme (i.e. catalysis of the reaction of lipohydroperoxides with other compounds) is involved in the respiratory inhibition of these sites (Schewe, T., Härtel, B., Hiebsch, C. and Rapoport, S.M., unpublished observations). It is also conceivable that the lipoperoxides formed by the action of the lipoxygenase on normal or Q-depleted particles prevent the reincorporation of native Q-10 either by change in the fluidity of the membrane phospholipids or by inactivation of Q-binding proteins [31]. Further studies are necessary to clarify the interactions between phospholipids, lipid peroxidation products, Q-10 and the interaction sites of Q-10 and the Fe-S clusters.

As far as the inhibition of cytochrome *c* oxidase is concerned, Wiesner et al. [30] have shown that it is relieved by subsequent addition of native phospholipids. Therefore, this inhibition has to be interpreted as a consequence of the attack of lipoxygenase on phospholipids which are known to be essential for the enzymic activity of cytochrome *c* oxidase. The lack of any effect on the EPR-detectable Cu and the cytochrome *a* upon lipoxygenase treatment, as found in this study, is fully in line with this interpretation.

The Fe-S clusters in the mitochondrial outer membranes are selectively destroyed by lipoxygenase treatment (Fig. 2). In these experiments, about 1 mg of the crude lipoxygenase was used per mg of submitochondrial particles. Under these conditions, about 15% of the acid-labile sulphur of the particles is lost (Fig. 3). If we use the values of Beinert [25] for the content of Fe-S cluster-containing complexes in submitochondrial particles, an estimate can be made of the expected loss in acid-labile sulphur content on destruction of the outer-membrane Fe-S clusters. Beef-heart submitochondrial particles contain per mg of protein: 0.09 nmol FMN from NADH dehydrogenase, containing 16–28 atoms of Fe and acid-labile sulphur per molecule of FMN, 0.04 nmol FAD from ETF * : Q oxidoreductase (4 Fe, S atoms), 0.2 nmol of covalently bound FAD from succinate dehydrogenase (6–8 Fe, S

* ETF, electron-transfer flavoprotein.

atoms), 0.46 nmol cytochrome c_1 from the cytochrome b-c_1 complex (2 Fe, S atoms) and 0.3 nmol Fe-S cluster from mitochondrial outer membranes (2 Fe, S atoms). Taking the lower values for Fe and acid-labile sulphur, as they agree best with quantitative EPR measurements [11,17–19], this gives $(0.09 \times 16) + (0.04 \times 4) + (0.2 \times 6) + (0.46 \times 2) + (0.3 \times 2) = 4.32$ natom of Fe and acid-labile sulphur per mg of protein. Destruction of the Fe-S clusters in the outer membrane will thus result in a loss of $0.6/4.32 = 14\%$ in acid-labile sulphur, which is very close to the value actually found (Fig. 3). Somewhat greater losses may be the result of partial destruction of the [4Fe-4S] cluster of succinate dehydrogenase as observed after longer incubations with increasing amounts of lipoxygenase.

It is noteworthy that, although the Fe-S clusters of the mitochondrial outer membranes are completely destroyed by lipoxygenase treatment (Fig. 2), no effect is seen on the rotenone-insensitive NADH : cytochrome c oxidoreductase activity (Table I) which also resides in the outer membrane [16]. This activity was also not inhibited in lipoxygenase-treated particles by the subsequent addition of the lipoxygenase inhibitor salicylhydroxamic acid which excludes the possibility that lipoxygenase itself can replace the Fe-S clusters in this reaction. This proves that the Fe-S clusters in the outer membranes are not functional in this activity in contrast to what has been suggested [23].

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