Co-oxidation of NADH and NADPH by a mammalian 15-lipoxygenase: inhibition of lipoxygenase activity at near-physiological NADH concentrations

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The purified 15-lipoxygenase from rabbit reticulocytes is capable of oxidizing NADH in the presence of linoleic acid and oxygen. This co-oxidation proceeds at a rate that amounts to approx. 7% of linoleic acid oxygenation rates. Although NADH inhibits the lipoxygenase reaction with linoleic acid as substrate (46% inhibition at 0.2 mM NADH), the reaction specificity of the enzyme was not altered since (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid was identified as the major reaction product. NADH oxidation was inhibited by NAD^+ (uncompetitive with respect to linoleate and mixed/competitive with respect to NADH), and NADPH or NMNH could substitute for NADH with slightly different apparent K_m values. NADH oxidation was enhanced at lower oxygen tension, but was completely prevented under anaerobic conditions. Computer-assisted modelling of 15-lipoxygenase/NADH interaction and sequence alignments of mammalian lipoxygenases with NADH-dependent enzymes suggested that there is no specific binding of the coenzyme at the putative fatty acid-binding site of lipoxygenases. These results suggest that NAD(P)H might be oxidized by a radical intermediate formed during the dioxygenase cycle of the lipoxygenase reaction but that NADH oxidation might not proceed at the active site of the enzyme. The mechanism and possible biological consequences of 15-lipoxygenase-catalysed NAD(P)H oxidation are discussed.

INTRODUCTION

Lipoxygenases (EC 1.13.11.33) comprise a family of plant and mammalian enzymes that catalyse the dioxygenation of polyunsaturated fatty acids to their corresponding $n-6$ hydroperoxy derivatives [1,2]. Although a variety of biologically relevant effects of 15-lipoxygenases and their reaction products have been reported (reviewed in [3]), no general concept for the physiological and/or pathophysiological role of these enzymes has been worked out. Unlike 5-lipoxygenases, which exhibit a high specificity for free arachidonic acid, 15-lipoxygenases are capable of oxygenating phospholipids [4] and biomembranes [5–7] without the preceding action of lipid-hydrolysing enzymes. In rabbit reticulocytes, mitochondrial membranes seem to be the preferred lipoxygenase substrate [8]. This finding, along with the biological dynamics of the enzyme, have implicated the 15-lipoxygenase in the breakdown of mitochondria during erythrocyte maturation [9].

Intracellular 15-lipoxygenase activity is regulated at several pre- and post-transcriptional levels. In immature rabbit reticulocytes, lipoxygenase translation is prevented by a 15-lipoxygenase mRNA-binding protein [10]. This protein seems to be degraded at later maturation stages, allowing the enzyme to be expressed. In human peripheral monocytes, which do not express the enzyme constitutively, lipoxygenase synthesis is induced when the cells are cultured in the presence of interleukins 4 or 13 [11,12]. Several lines of evidence suggest that the modulation of cellular peroxide tone might have an important role in the regulation of intracellular 15-lipoxygenase activity. Many years ago it was shown that lipoxygenases require small amounts of peroxides for activation [13,14]. The preincubation of complex 15-lipoxygenase substrates such as biomembranes and lipoproteins with the phospholipid hydroperoxide glutathione peroxidase, which reduces hydroperoxy lipids present in the substrates, completely blocked 15-lipoxygenase-catalysed oxygenation [15]. Translating these findings to situations *in vivo* one can hypothesize that the redox status of the cell might be an important regulator of intracellular 15-lipoxygenase activity.

NADPH and NADH have central roles in most bioenergetic and biosynthetic processes as intracellular carriers of reducing equivalents, and thus the ratio of NAD(P)H to $NAD(P)^+$ can be regarded as an indicator of cellular redox status. Although redox reactions of both compounds are usually mediated by two-electron hydride transfer, one-electron oxidation in the presence of reducing agents and oxygen has also been described [16–19]. During such reactions, which are mainly catalysed by peroxidases or haemoproteins, NADH is oxidized to $NAD^{\cdot}$, a potent reductant ($E_{m,7.0}^{NAD+/NAD^{\cdot}} = -920$ mV [20]) capable of reacting rapidly with oxygen ($1.9 \times 10^9\ M^{-1} \cdot s^{-1}$) to form a superoxide anion [21]. Previous studies have shown that the soybean lipoxygenase, which differs from mammalian 15-lipoxygenases with respect to its protein chemical and enzymic properties, co-oxidizes NAD(P)H during the oxygenation of linoleic acid [22,23].

The results presented here indicate that a mammalian 15-lipoxygenase is capable of catalysing NAD(P)H oxidation in the presence of polyenoic fatty acids and oxygen. The inhibition of the oxygenase activity by NAD(P)H and its counteraction by NAD^+ suggests that the cytosolic ratio of NADH to NAD^+ might play a role in the regulation of cellular 15-lipoxygenase activity.

MATERIALS AND METHODS

Materials

The rabbit reticulocyte lipoxygenase was purified to electro-

Abbreviations used: ABAP, 2,2′-azobis-(2-amidinopropane) dihydrochloride; HPODE, hydroperoxyoctadecadienoic acid.

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phoretic homogeneity from the lysate of a reticulocyte-rich blood cell suspension by fractionated $(NH_4)_2SO_4$ precipitation and two consecutive steps of FPLC [24]. Unless otherwise stated, all enzymes and chemicals used for this study were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 2,2′-Azo-bis-(2-amidinopropane) dihydrochloride (ABAP) was obtained from Wako Chemicals (Richmond, VA, U.S.A.). (13*S*)-Hydroxyperoxy-(9*Z*,11*E*)-octadecadienoic acid [(13*S*)-HPODE] was obtained from Cascade (Reading, Berks., U.K.).

Assay systems

Lipoxygenase activity was assayed either spectrophotometrically measuring the increase in absorbance at 234 nm (using an ϵ_{234} of 28 $mM^{-1} \cdot cm^{-1}$ for conjugated dienes), or oxygraphically using a Clark-type oxygen electrode and a pO_2 meter (YSI 35). The lipoxygenase assay was performed at 20 °C and the assay mixture was a 0.1 M phosphate buffer (pH 7.4) containing linoleic acid (0.575 mM final concentration) and 0.2 % sodium cholate as detergent [25]. To remove adventitious metal ions the phosphate buffer was pretreated with chelating resin [26]. Lipoxygenase-catalysed hydroperoxidase activity and NAD(P)H oxidation were assayed spectrophotometrically by recording the absorbances at 285 and 340 nm respectively. NADH oxidation rates were calculated from an ϵ_{340} of 6.22 $mM^{-1} \cdot cm^{-1}$. Oxygen concentrations of air-saturated buffers at 20 °C were determined with hydrogen peroxide and catalase. Measurements at various oxygen concentrations were performed after mixing different volumes of aerobic and anaerobic buffers in a gas-tight cuvette. Ascorbate oxidation was monitored at 265 nm with an ϵ_{265} of 7 $mM^{-1} \cdot cm^{-1}$.

Non-enzymic linoleate oxidation

Linoleic acid (0.57 mM final concentration) was dissolved in 0.1 M phosphate, pH 7.4, containing 0.2 % sodium cholate and was oxidized at 20 °C by the addition of ABAP (up to 1 mM) in the presence of NADH (0.1 mM); the absorbance was monitored at 234 and 340 nm. ABAP was freshly made in phosphate buffer (0.1 M, pH 7.4) and kept on ice until use.

HPLC analysis of reaction products

The lipoxygenase-catalysed oxidation of linoleic acid in the presence and in the absence of NADH were stopped by acidification to pH 3, and lipids were twice extracted with 2 vol. of diethyl ether. The extracts were dried over sodium sulphate (30 min at 4 °C) and the solvent was evaporated under vacuum. The remaining lipids were reconstituted in 0.2 ml of methanol and stored at −80 °C under nitrogen atmosphere. Normal-phase HPLC was performed on a Shimadzu instrument coupled with a Hewlett Packard diode array detector 1040. The hydroxy fatty acid isomers were separated on a Zorbax SIL column (DuPont; 250 mm × 4.6 mm, 5 μm particle size) with a solvent system of n-hexane/propan-2-ol/acetic acid (100:2:0.1, by vol.) and a flow rate of 1 ml/min. For analysis of the enantiomer composition the hydroperoxy fatty acids were reduced with triphenylphosphine and the resulting hydroxy fatty acids were analysed by chiral-phase HPLC [27] on a Chiralcel OD column (Diacel Chemical Industries, 250 mm × 4.6 mm, 5 μm particle size) with a solvent system of hexane/propan-2-ol/acetic acid (100:5:0.1, by vol.).

Kinetic and statistical analysis

Kinetic analysis was performed with Enzfitter (R. J. Leatherbarrow, Elsevier Biosoft, 1987). Statistical analysis was performed by one-way analysis of variance with the Bonferroni *post hoc* test (Statgraphics 3.0, Rockville, MD, U.S.A.). $P < 0.05$ was considered to indicate a statistically significant difference. Regression analysis was undertaken by calculation of Pearson product moment correlation coefficient (Microsoft Excel, version 7.0).

RESULTS

NAD(P)H oxidation by 15-lipoxygenases

In the presence of linoleic acid (0.575 mM final concentration) and oxygen, the rabbit reticulocyte lipoxygenase [linoleic acid oxygenase activity 13.3 ± 0.9 (mean ± S.D.) μmol/min/mg, n = 3] exhibited NADPH and NADH oxidase activities of 583 and 691 nmol/min per mg respectively (Figure 1). As for the lipid dioxygenase activity, oxidation of NAD(P)H proceeded for approx. 3–4 min and then ceased because of self-inactivation of the enzyme (a poorly understood process associated with oxidation of an active-site methionine by hydroperoxide product, which occurs 2–3 min after mixing of enzyme and fatty acid substrate [28]) (Figure 1a). Neither linoleic acid, nor oxygen or NADH were exhausted during the incubation period. When linoleic acid was omitted from the reaction mixture no NADH oxidation was observed (results not shown). In the presence of near-physiological concentrations of NADH (0.2 mM), a 42 %

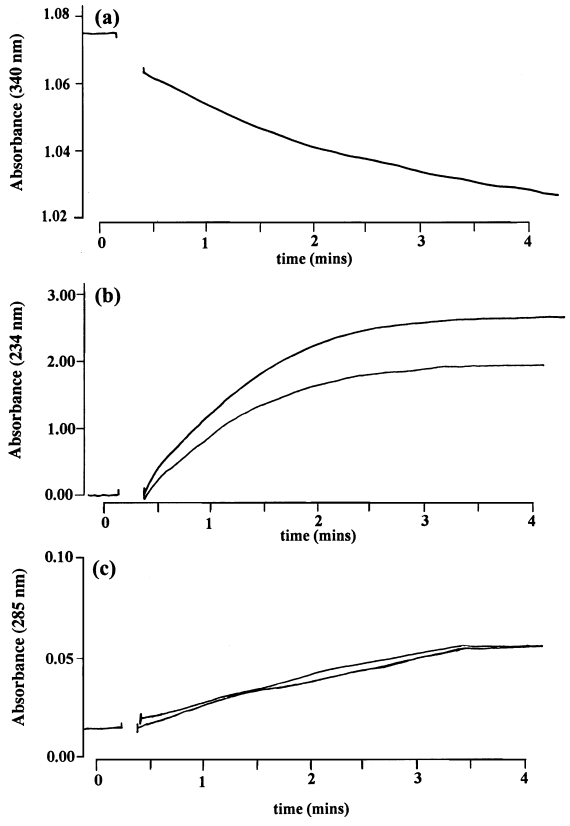


Figure 1 Progress curves for NADH oxidation, diene conjugation and peroxidase activity by reticulocyte 15-lipoxygenase

Enzyme (3.5 μg) was added to 1 ml of Chelex-treated phosphate buffer (0.1 M, pH 7.4) containing linoleate (575 μM) and 0.2% sodium cholate at 20 °C with or without NADH (200 μM). Absorbance was monitored at (**a**) 340 nm for NADH oxidation, (**b**) 234 nm for diene conjugation [upper trace, control; lower trace, NADH (100 μM)] and (**c**) 285 nm for peroxidase activity [control and NADH (200 μM) shown].

Table 1 Effect of NADH and NAD^+ on diene conjugation by lipoxygenases

Rabbit enzyme (2 μg) or soybean enzyme (5 μg) was added to 1 ml of phosphate buffer (0.1 M, pH 7.4) containing linoleate (575 μM) and sodium cholate (0.2%) with or without NADH or NAD^+, and rates of diene conjugation were monitored at 234 nm ($n = 3$, means $\pm$ S.D.). Statistical analysis was by one-way ANOVA with the Bonferroni *post hoc* test. * Statistically significant difference from control, with $P < 0.05$.

Lipoxygenase	Addition	Diene conjugation rate (μmol/min per mg of enzyme)
Rabbit	None (control)	16.4 $\pm$ 0.54
	100 μM NADH	11.1 $\pm$ 0.38*
	200 μM NADH	9.7 $\pm$ 0.48*
	100 μM NAD^+	18.7 $\pm$ 0.82
	200 μM NAD^+	19.7 $\pm$ 0.57*
	100 μM NAD^+ + 100 μM NADH	12.75 $\pm$ 1.02*
Soybean	None (control)	10.26 $\pm$ 0.22
	100 μM NADH	8.88 $\pm$ 0.14
	200 μM NADH	8.26 $\pm$ 0.28

Table 2 Calculation of K_m (app) for NAD(P)H/NMNH and linoleate for 15-lipoxygenases

Rabbit or soybean enzyme was added to 1 ml of Chelex-treated phosphate buffer (0.1 M, pH 7.4) containing linoleate (varying), NAD(P)H/NMNH (varying) and 0.2% sodium cholate at 20 °C. Absorbance was monitored at 340 nm for pyridine nucleotide K_m calculations or 234 nm for linoleate K_m calculations. K_m (app) means $\pm$ S. E. M. were determined by non-linear fit to the Michaelis-Menten equation by using Enzfitter (Biosoft). Abbreviation: n.d., not determined.

	K_m (app) (μM)	
Varying substrate	Rabbit reticulocyte lipoxygenase	Soybean lipoxygenase
NADH	160 $\pm$ 19.2	17.1 $\pm$ 2.0
NADPH	85.1 $\pm$ 21	53.3 $\pm$ 3.9
NMNH	72 $\pm$ 9	231 $\pm$ 5.9
Linoleate (diene conjugation)	7.87 $\pm$ 1.09	n.d.
Linoleate (diene conjugation + 200 μM NADH)	5.64 $\pm$ 1.11	n.d.

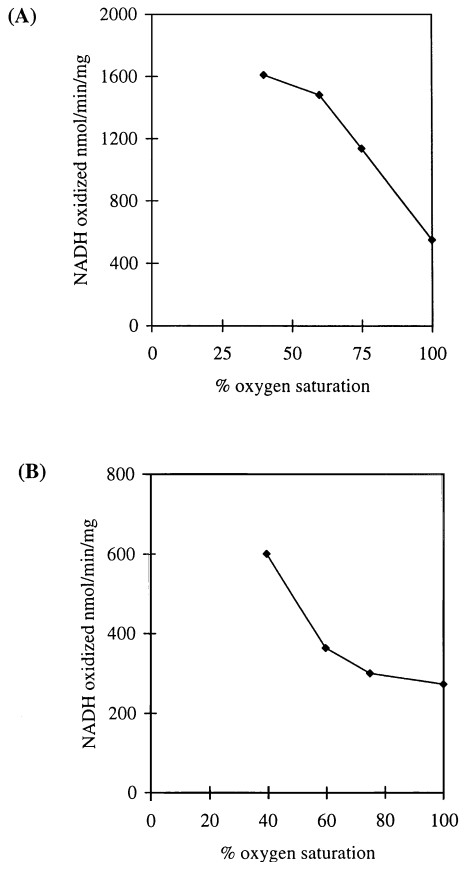


Figure 2 Effect of varying air saturation on the rate of NADH oxidation by lipoxygenase

The air saturation of samples was varied by mixing aerobic and anaerobic buffers together in the anaerobic cuvette as described in the Materials and methods section. Rabbit enzyme (1.75 μg) (**a**) or soybean enzyme (10 μg) (**b**) was added to 1 ml of Chelex-treated phosphate buffer (0.1 M, pH 7.4) containing 575 μM linoleate, 100 μM NADH and 0.2% sodium cholate at 20 °C and the rate of NADH oxidation was monitored at 340 nm. An air saturation of 100% corresponded to an oxygen concentration of 0.235 mM.

inhibition of the linoleic acid oxygenase activity was observed, as indicated by a decrease in the formation of conjugated dienes (Table 1) and by HPLC quantification of the major oxygenation product (13*S*)-HPODE (results not shown). This inhibition could be slightly reversed by the simultaneous addition of NAD^+ (Table 1). Also, the addition of NAD^+ alone led to dose-dependent increases in dioxygenase activity. In contrast, the lipohydroperoxidase activity of the enzyme measured as the formation of conjugated ketodienes (increase in absorbance at 285 nm) was not affected (Figure 1c). A similar NAD(P)H oxidation was observed for soybean lipoxygenase 1, which was used as a control enzyme (results not shown). Linoleic acid oxygenase activity of the soybean enzyme was also inhibited by NADH but the extent of inhibition was considerably lower (Table 1).

Kinetic characterization of NAD(P)H oxidation

For basic kinetic characterization of lipoxygenase-catalysed NAD(P)H oxidation, apparent K_m values for linoleic acid, NADH and NADPH were determined using a non-linear fit to the Michaelis–Menten equation (Table 2). For the rabbit enzyme, the apparent K_m for linoleic acid was not altered in the presence of 200 μM NADH and was not significantly different from previously reported values (6.36 μM [29]). Comparison of the apparent K_m values for NADH and NADPH indicates small differences in the specificities of both enzymes for reduced pyridine nucleotide substrates. To determine specificity further, we examined for the oxidation of two additional potential substrates, NMNH (an NADH analogue that lacks the AMP moiety) and the peroxyl radical scavenger ascorbate. NMNH was oxidized immediately on enzyme addition, in a manner identical to that of both NADH and NADPH (Table 2); however, the oxidation of ascorbate (200 μM) showed a lag phase of approx. 1 min (results not shown).

Mechanistic studies

To elucidate the mechanism of lipoxygenase-mediated NADH oxidation two major questions needed to be addressed: (1) What is the chemical nature of the NAD(P)H oxidizing species? (2) Where does NAD(P)H oxidation take place: at the putative substrate-binding site of the enzyme or outside the active site, by an oxidizing intermediate of the oxygenase reaction that has dissociated?

To address the first question we performed experiments under

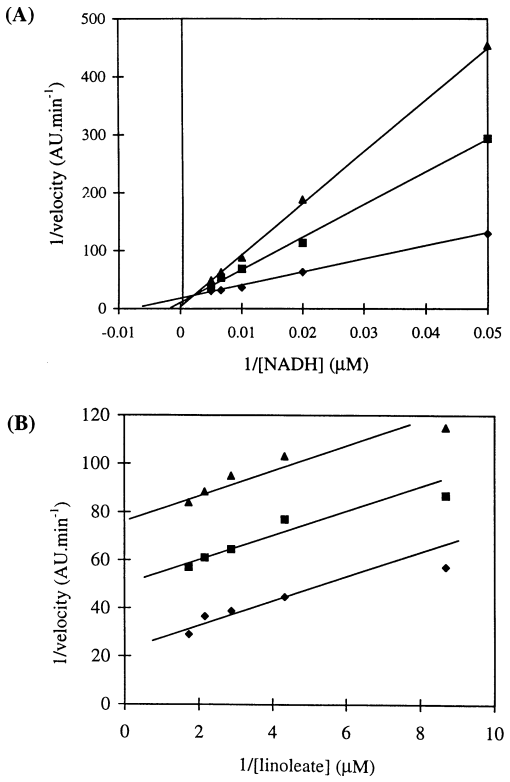


Figure 3 Inhibition of 15-lipoxygenase-mediated NADH oxidation by NAD^+

Rabbit enzyme (3.5 μg) was added to 1 ml of Chelex-treated phosphate buffer (0.1 M, pH 7.4) with 0.2% sodium cholate at 20 °C. (**a**) Reaction mixture containing 575 μM linoleate, varying NADH. Symbols: ◆ , control, $r = 0.99$; ■, 400 μM NAD^+, $r = 0.99$; ▲, 800 μM NAD^+, $r = 0.99$, (**b**) Reaction mixture containing 200 μM NADH, varying linoleate. Symbols: ◆ , control, slope 3.6, $r = 0.97$; ■, 400 mM NAD^+, slope 4.1, $r = 0.96$; ▲, 800 μM NAD^+, slope 4.2, $r = 0.96$. Rates of NADH oxidation [absorbance units (AU)/min] plotted against [NADH] (μM) or [linoleate] (μM) with or without NAD^+ were plotted as double reciprocals. r is the Pearson product moment correlation coefficient.

decreased oxygen tensions. As shown in Figure 2, NADH oxidation was enhanced at lower oxygen concentrations, suggesting that the formation of the NADH-oxidizing species is stimulated under these conditions. In contrast, we observed a total suppression of NADH oxidation under anaerobic conditions even if hydroperoxylinoleic acid was added (results not shown). These results suggest that the oxidizing species is formed during the oxygenase reaction and that the anaerobic hydroperoxidase activity of lipoxygenases might not be involved.

In the presence of fatty acid substrates, 15-lipoxygenases are capable of catalysing the oxidation of reducing agents (RH) such as *N*-hydroxyureas, hydroxybenzofurans and hydroxamic acids [30–32]. It has been suggested that the oxidation of these is catalysed by the ferric enzyme to give $R^{\bullet}$ and the ferrous enzyme. Regeneration of the oxidized enzyme is accomplished by homolytic cleavage of the peroxy bond forming an alkoxy radical ($LO^{\bullet}$) and OH^-. However, the addition of (13*S*)-HPODE (0.16 mM) instead of linoleic acid did not result in NADH oxidation. Moreover, there was no effect of (13*S*)-HPODE on the rate of linoleic acid-dependent NADH oxidation (results not shown). These results suggest that NAD(P)H might be oxidized by an intermediate that is formed before (13*S*)-HPODE during the oxygenase cycle and that the ferric enzyme itself might not be the oxidant.

NAD^+, the product of NADH oxidation, was shown to inhibit lipoxygenase-mediated NADH oxidation. Double-reciprocal plots of substrate concentration against NADH oxidation suggested an uncompetitive inhibition with respect to linoleic acid (Figure 3b) but a mixed/competitive type of inhibition with respect to NADH (Figure 3a). This behaviour suggests that NAD^+ displaces NADH from the site of oxidation.

To address the second mechanistic question we performed sequence alignments and computer-assisted modelling studies. Pyridine nucleotide-binding enzymes contain short glycine-rich sequences that indicate the cofactor-binding regions [33–36]. We attempted to align several nucleotide-binding motifs with the amino acid sequences of plant and mammalian lipoxygenases but did not find any obvious sequence similarities. Furthermore we constructed a three-dimensional model of the putative substrate-binding regions of both the rabbit and the soybean lipoxygenase on the basis of the X-ray coordinates for the soybean crystals [37] and attempted to fit in NADH. We found that there is not enough space available for specific NADH binding unless the three-dimensional structure of the enzyme is seriously altered. These findings suggest that NADH oxidation might not proceed at the substrate-binding site of the enzyme.

Superoxide anions and free peroxylipid radicals might not be involved in lipoxygenase-dependent NADH oxidation

It has been suggested [38] that superoxide might be involved in the propagation of NADH oxidation by peroxidases. If a similar mechanism is involved in 15-lipoxygenase-mediated NADH oxidation, inhibition by superoxide scavengers should be observed. We found that superoxide dismutase (0.02 mg/ml) did not exhibit any effect on NADH oxidation (results not shown). This is a strong argument against the involvement of the free superoxide anion in lipoxygenase-dependent NADH oxidation. For the soybean lipoxygenase it has been reported [22,23] that superoxide is formed during NADH oxidation in the presence of linoleic acid and oxygen. This conclusion was drawn from the superoxide dismutase-inhibited reduction of cytochrome *c*. Using this methodology we were unable to detect superoxide generation either with the soybean lipoxygenase or with the rabbit enzyme. In our hands, the addition of ferricytochrome *c* to assay mixtures containing the lipoxygenase, linoleic acid, oxygen and NADH led to huge increases in NADH oxidation. For example, the addition of cytochrome *c* (0.05 mM) to the soybean lipoxygenase assay containing 0.29 mM linoleic acid and 0.2 mM NADH led to a 43-fold increase in NADH oxidation (32 nmol/min without ferricytochrome *c*, compared with 1.38 mmol/min after ferricytochrome *c* addition). It is likely that the increase in NADH oxidation is a consequence of cytochrome *c*-catalysed decomposition of 13-HPODE formed via the lipoxygenase reaction. During this reaction, oxidizing radical intermediates such as alkoxyl radicals and the hydroxyl radical might be formed [39], which might oxidize NADH.

Under normoxic conditions, the oxidation of naturally occurring polyenoic fatty acids is completely enzyme-controlled. Radicals formed during the oxygenase cycle remain enzyme-bound and only the hydroperoxy product is released from the enzyme. The specific pattern of oxygenation products can be regarded as an indicator of the high degree of control. However, at low oxygen concentrations (30 μM or less) a small percentage (up to 10%) of the fatty acid radicals formed during initial hydrogen removal escape the active site of the lipoxygenase (H. Kühn and P. Ludwig, unpublished work). These react with oxygen at diffusion-controlled rates (3×10^8 $M^{-1} \cdot s^{-1}$) to form free lipid peroxyl radicals ($LOO^{\bullet}$). Because this oxygen insertion is no longer controlled by the lipoxygenase, a proportion of

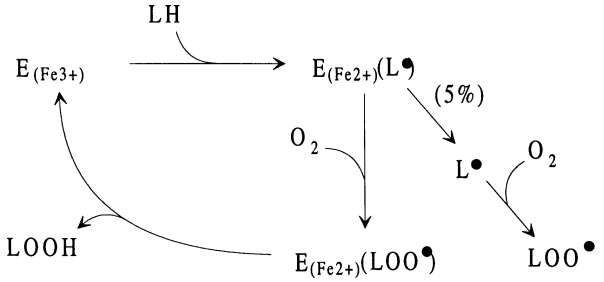
racemic reaction products are formed. To determine whether free LOO• can oxidize NAD(P)H, we replaced lipoxygenase with the azo compound ABAP and examined for NADH oxidation during ABAP-induced linoleic acid oxidation. However, we were unable to detect any NADH oxidation even under conditions where the steady-state concentrations of the LOO• radical were estimated to be much higher than in the lipoxygenase assay. To characterize free radical intermediates present in our system we attempted to spin-trap them with either 5,5′-dimethyl-1-pyrroline *N*-oxide or 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide. Unfortunately, both spin traps significantly inhibited NADH oxidation (results not shown).

DISCUSSION

In this study we show that a mammalian 15-lipoxygenase is capable of co-oxidizing reduced pyridine nucleotides in the presence of polyenoic fatty acid substrate and oxygen. At near-physiological NADH concentrations (mitochondrial and cytosolic NADH at 0.27 and 0.64 mM respectively [40]) a marked inhibition of the fatty acid oxygenase activity of a mammalian lipoxygenase was observed. Interestingly, this inhibition could be partly counteracted by NAD^+. Thus the cellular redox potential (ratio of NADH to NAD^+) might be a physiological regulator of the cellular 15-lipoxygenase activity. In fact, previous studies have shown that energized mitochondria, which are characterized by a high ratio of NAD(P)H to $NAD(P)^+$, are resistant to 15-lipoxygenase attack [6,41].

In cellular systems 15-lipoxygenases are an effective source of hydroperoxy lipids. These rather unstable products are either reduced to more stable hydroxy lipids via glutathione-dependent pathways or converted via free radical chain reactions to an array of secondary products of lipid peroxidation. *In vivo* such non-enzymic secondary reactions utilizing peroxylipids provided by 15-lipoxygenases might have an important role. There are several lines of experimental evidence suggesting the involvement of such secondary reactions in 15-lipoxygenase/membrane interaction. (1) The oxygenation of mitochondrial membranes at high substrate concentrations *in vitro* leads to an unspecific pattern of oxygenation products [7]. If the reaction is performed in the presence of a peroxide-reducing enzyme (phospholipid hydroperoxide glutathione peroxidase), a specific product pattern is retained [15], suggesting that the decomposition of specific lipoxygenase products might be responsible for the non-specificity. (2) Oxygen uptake during lipoxygenase reaction with mitochondrial membranes far exceeds the rate of hydroperoxy-lipid formation [42], suggesting lipoxygenase-dependent secondary reactions. (3) Mitochondrial membranes of rabbit reticulocytes contain a large share of non-specific lipid peroxidation products [8] that are unlikely to be formed directly by the 15-lipoxygenase. It is possible that cytochrome *c*, present in the intermembrane space, accelerates lipoxygenase-mediated oxidative damage via peroxidase-type reactions with (13*S*)-HPODE and NADH that are similar to those observed here. In support of this, a central role for cytochrome *c* in hydroperoxide-induced mitochondrial lipid peroxidation has been described by Radi et al. [43].

The chemical mechanism of 15-lipoxygenase-mediated NADH oxidation and the site where this reaction takes place remain unclear. We have shown that both fatty acid substrates and oxygen are required, but that hydroperoxy fatty acids are not essential. The K_m for oxygen for linoleate oxidation is very low (3.7 μM [14]). and is believed to result from the high affinity of the lipoxygenase–lipid alkyl radical complex for oxygen addition. However, other lipoxygenase-induced secondary reactions are influenced by oxygen concentration changes well above the K_m.



Scheme 1 15-Lipoxygenase dioxygenase cycle

Abbreviations: E, enzyme; LH, unsaturated fatty acid substrate; LOO•, lipid peroxyl radical; L•, lipid alkyl radical.

For example, at 30 μM, increased hydroperoxidase activity is observed, oxygen uptake and conjugated diene rates begin to deviate and changes in S/R ratio are observed [43a], unpublished work). Because NADH oxidation seems to be a secondary process, it might have different kinetic parameters from linoleate oxidation and thus be influenced by variations in oxygen tensions above the K_m. Although we do not understand the mechanism of oxygen stimulation of NADH oxidation, the absolute requirement for oxygen indicates that an intermediate of the dioxygenase cycle rather than of the peroxidase activity is likely to mediate this activity.

Oxidation of NADH requires a strong one-electron oxidant ($E_{m,7.0}^{NAD^•/NADH} = 282$ mV), and several candidates [44] are formed during the dioxygenase cycle (Scheme 1).

The oxidized enzyme (Fe^{3+}) is competent for aerobic one-electron oxidation of several reducing substrates [28–30]. However, the absolute requirement of NADH oxidation for unsaturated fatty acid (LH) rather than the peroxide product (LOOH) ruled out an involvement of the ferric enzyme. During the oxygenase cycle a fatty acid radical (L•) is formed from linoleic acid via stereoselective hydrogen abstraction. This radical is also unlikely to be the NAD(P)H oxidant because it is formed in the presence and in the absence of oxygen.

Our model studies on NADH binding to 15-lipoxygenases suggest that NADH oxidation might not take place at the substrate-binding region of the enzyme. Alternatively, radical intermediates of the oxygenase cycle might escape the substrate-binding region and oxidize NADH at the periphery of the enzyme or in the surrounding medium. However, in neither case would any specificity for NAD(P)H over ascorbate be expected. Under hypoxic conditions, the rate of lipid radical (L•) dissociation and formation of free LOO• seems to be increased as determined by the increased share of racemic oxygenation products. In addition, LOO• seems to be the only radical intermediate generated mainly during the aerobic dioxygenase cycle but not under anaerobic conditions [45]. However, our experiments with ABAP-initiated lipid oxidation demonstrate that free LOO• alone is insufficient to drive NADH oxidation by lipoxygenase.

Although we do not know the mechanism of NAD(P)H oxidation, several features suggest a direct role for the lipoxygenase enzyme in mediating this process: (1) the inhibition or stimulation of diene conjugation by reduced or oxidized pyridine nucleotides respectively suggests a direct interaction between NAD(P)H and enzyme; (2) the inhibition of NAD(P)H oxidation by NAD^+ would not be expected in a purely chemical system; (3) free-radical intermediates in solution would be expected to oxidize ascorbate with similar kinetics to that for NAD(P)H.

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