Iron Release from the Active Site of Lipoxygenase
Claus Fuchs and Gerhard Spiteller*
Institut für Organische Chemie I, Universität Bayreuth, Universitätstr. 30, 95440 Bayreuth, Germany. Fax: 0921/552671. E-mail: gerhard.spiteller@uni-bayreuth.de
* Author for correspondence and reprint requests
Z. Naturforsch. 55c, 643–648 (2000); received March 23/April 17, 2000
Lipoxygenase, Iron Release, Myocardial Infarction

In the course of the lipoxygenase-catalyzed transformation of linoleic acid to 13S-hydroperoxy-9Z,11E-octadecadienoic acid, iron ions are liberated. This iron release has been determined using a spectrophotometric assay based on the complexation of ferrous iron by 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine disodium salt (ferrozine). Further comparative measurements demonstrated that iron release correlates to deficient oxygen supply. We speculate that release of iron ions is caused by modifications of histidine residues located at the active site of the enzyme. Liberation of iron ions may be responsible for increased generation of lipid peroxidation (LPO) products observed after a myocardial infarction since iron ions induce nonenzymic LPO processes.

Introduction
Inflammatory processes, for instance rheumatoid arthritis, but also atherosclerosis, are characterized by cell injury and cell degradation: Any cell injury activates lipases and lipoxygenases (Spiteller, 1996): lipases cleave cell membrane phospholipids and thus generate free polyunsaturated fatty acids (PUFAs). PUFAs are the substrates for lipoxygenases (Yamamoto, 1992). Lipoxygenase (LOX) (EC 1.13.11.12) catalyzes the dioxygenation of fatty acids which contain one or more 1(Z),4(Z)-pentadiene systems yielding chiral dioxygenation of fatty acids which contain one or more 1(Z),4(Z)-pentadiene systems yielding chiral (E,Z) conjugated hydroperoxy fatty acids (LOOHs) (Veldink and Vliegenthart, 1984).

LOOHs are transformed in biological surrounding to corresponding alcohols (LOHs). In this reaction the stereochemistry is retained. Therefore one expects to find in diseases connected with cell damage chiral LOHs. Investigation of the stereochemistry of hydroxy groups in LOHs isolated from patients revealed that most but not all LOHs are racemic: Racemic LOHs are generated in nonenzymic LPO processes (Kühn, 1994; Folcik et al., 1995), induced by bivalent metal ions which react with LOOHs to generate in a Fenton type reaction according to the equation (1) and (2) (Koppenol, 1994) LOO* and LO* radicals.

L-O-O-H + Fe+++ → LO* + OH− + Fe++ (1)
L-O-O-H + Fe+++ → LOO* + H+ + Fe++ (2)

Radicals are able to remove a hydrogen radical from a double allylically activated CH2 group of a PUFa. The thus generated mesomeric radical reacts with oxygen, producing two regio-isomeric peroxyl radicals which in turn remove hydrogen from another PUFa, thus starting a chain reaction (Frankel, 1985) (Scheme 1):

The nonenzymic LPO observed in atherosclerosis and other diseases requires therefore presence of iron ions which transform LOOH, generated after cell injury by lipoxygenases, to radicals which then induce the autocatalytic LPO.

Fe-ions are usually not detectable in biological material, although Fe-ions are essential components of blood and many enzymes, including lipoxygenases. In lipoxygenases and other iron containing enzymes, iron ions are shielded, they are not able to induce cleavage of hydroperoxides to radicals (Aust et al., 1985). 1986 Gutteridge (Gutteridge, 1986) demonstrated that free radicals generated by excess oxidants release heme-bound...
iron (Rice-Evans et al., 1989) from hemoglobin and myoglobin. As radicals are generated in the course of catalytic turnover of linoleic acid / LOX too, it was hypothesized that iron may analogously be released from the active site of LOX. Verification of this assumption is reported in this paper.

**Experimental**

**Materials**

Lipoxygenase, linoleic acid, ascorbic acid, ferrozine, TRIS, HEPES, and neocuproine were purchased from FLUKA, Neu-Ulm (Germany). Boric acid and ammonium acetate were from Merck, Darmstadt (Germany).

**UV spectroscopy**

UV measurements were performed with a SHIMADZU UV-160A UV-VISIBLE spectrometer.

**Freeze drying** was performed with a Christ® alpha II – 12.

**Iron release from LOX**

16.7 μl linoleic acid was suspended in 500 ml borate buffer (0.2 mM, pH 9) respectively 500 ml HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer (50 mM, pH 7.4) and vigorously stirred. In order to obtain reproducible starting conditions, the suspension was once saturated with oxygen by discharging O₂ through the suspension for one min (22 °C; 5 x 10⁴ Pa). The resulting oxygen concentration was reported to be 240 μM at room temperature (De Groot et al., 1975). After addition of 1.9 mg lipoxygenase (7.1 U/mg), the solution was stirred for one hour at room temperature under permanent discharging of oxygen (aerobic conditions) respectively under argon (anaerobic conditions). This solution was acidified by addition of conc. HCl until pH 2 was reached and immediately frozen by liquid nitrogen. Sample concentration was achieved by freeze drying. Then the samples were redissolved in 5 ml 0.2 N HCl, centrifuged (3350 g) and filtered (0.2 μm).

Since oxygen is a biradical, the ground level of iron release from LOX by pure oxygen was determined (22 °C; 5 x 10⁴ Pa; 1h; no linoleic acid).

**Iron determination** (Carter, 1971; Fish, 1988) 500 μl of each sample were incubated for 5 min with 500 μl of the reducing agent (0.02% ascorbic acid in 0.2 N HCl). After further addition of 500 μl 10% ammonium acetate solution and 200 μl ferrozine solution (75 mg ferrozine and 75 mg neocuproine in 25 ml of water acidified with one drop of conc. hydrochloric acid) the absorbance was measured at 562 nm after 48 h.

Since it was shown that even purified LOX was contaminated by traces of copper (De Groot, 1975), the addition of neocuproine complexing copper traces was necessary.

For each sample, a blank was prepared by substituting the ferrozine/neocuproine solution by 200 μl of water.

Expectedly, substitution of the reducing agent by 0.2 N hydrochloric acid proved that ferric iron was present in the solution at the applied conditions.

Panter (1994) already demonstrated that in absence of buffering capacity or in presence of physiological saline, iron release is attenuated, but iron release is increased in presence of TRIS (tris(hydroxymethyl)aminomethane hydrochloride) or phosphate buffer. These results were confirmed. Probably TRIS and phosphate are capable of interacting directly with iron removing it from the enzyme-binding site and increasing its solubility.

For this reason, control experiments were performed incubating LOX in borate-respectively HEPES buffer without linoleic acid addition (conditions and processing as presented above). These studies proved that within the range of sensitivity of the applied assay borate buffer did not show...
any iron release. Nevertheless, HEPES contained a small, yet highly reproducible, amount of iron ions.

**Results and Discussion**

Since Stookey introduced ferrozine to determine iron ions in biological fluids (Stookey, 1970), this reagent has been used extensively as a chromophore to measure iron ions in a variety of assays (Carter, 1971; Fish, 1988). Carter's original ferrozine assay for serum iron ions (Carter, 1971) has proven to be reliable, but perhaps less sensitive than the bleomycin assay for free iron ions (Panter, 1994). However, numerous compounds interfere with the latter assay thus reducing its applicability (Fish, 1988).

For that reason the iron release from soybean LOX I was studied with the ferrozine assay, based on the complexation of bivalent iron ions by ferrozine (Carter, 1971).

Previous studies indicate that agents that either interact with or bind iron ions, i.e., fatty acids (Nalini and Balasubramanian, 1993; Ni et al., 1997), may significantly influence iron ion recovery from solutions (Panter, 1994). Therefore further experiments were performed in order to determine iron ions in presence of different concentrations of linoleic acid (Fig. 1).

These control experiments revealed that iron ion determination by the ferrozine assay is proportional to the amount of linoleic acid in the solution. This effect may be contribute to the siderophoric activity of lipids, i.e., fatty acids (Nalini and Balasubramanian, 1993; Ni et al., 1997) complexing released LOX-iron. Nevertheless, Figure 1 shows that this iron ion complexation by linoleic acid is fully reversible after 48 h. In consequence, absorbance was measured 48 h (not 15 min (Carter, 1971; Fish, 1988)) after ferrozine addition.

Similar results were obtained in experiments using 1 μM, 10 μM and 100 μM solutions of FeSO₄ • 7 H₂O.

Figure 2 represents the iron ion release from LOX caused by its own substrate linoleic acid, respectively secondary “reactive oxygen species (ROS)” and free radicals generated by aerobic and anaerobic conditions.

As oxygen, a biradical, causes iron ion release itself and HEPES is contaminated by a small amount of iron ions (< 0.1%, according to Fluka) blank measurements were necessary. For that reason, iron ion release induced by oxygen, respectively, the basic iron ion level in HEPES buffer were determined (3 and 4 in Fig. 2) and the absorbance (1 and 2 in Fig. 2) appropriately corrected (5 and 6 in Fig. 2).

Iron ion release from LOX at anaerobic conditions (6 in Fig. 2) exceeds that at aerobic conditions (5 in Fig. 2) for a factor of about 2.5. The iron ion release experiments obtained at the pH-optimum of soybean LOX I (pH = 9.0, 0.2 M borate buffer; 18 μM linoleic acid; 0.016 U/ml LOX), n = 4) are described shortly as follows:

![Fig. 1. Time-dependent ferrous iron determination in presence of different concentrations of linoleic acid.](image-url)
If only LOX is stirred with oxygen the mean absorbance at 562 nm is 0.0425 (SD = 0.00289). In this case surprisingly, iron ion release by oxygen exceeds that one caused by incubation of LOX with linoleic acid and oxygen (mean absorbance at 562 nm is 0.01825 ± 0.00419). Incubation of LOX and linoleic acid without oxygen results in a mean absorbance at 562 nm of 0.07025 ± 0.00206. This result is consistent with previous studies demonstrating that the presence of either 13-HPODE (for soybean LOX) or 9-HPODE (for tomato LOX) protects the enzyme against denaturation (Perez-Gilabert et al., 1996). This reduction of the iron release may be due to shielding of the active site by reduced access of damaging ROS to susceptible histidine residues.

The primary structure of LOX-1 has been fully determined (Shibata et al., 1987): three histidines and one isoleucine as well as one iron atom are located at the active site (De Groot et al., 1975; Percival, 1991). This iron is essential for catalytic activity (Percival, 1991).

With respect to Cu,Zn-superoxide dismutase (SOD) it has been ascertained by various studies (Salo et al., 1990) that it is inactivated by its own reaction product, concurrent with the generation of OH* (Uchida and Kawakishi; 1994, Yim et al., 1993) copper release and enzyme inactivation, respectively.

Due to the presence of three labile histidines (Uchida and Kawakishi, 1994; Zhao et al., 1997) LOX may be similarly affected by active site modifications causing iron release and LOX inactivation (Percival, 1991).

It is important to recognize that the normal aerobic LOX cycle is in constant competition with the anaerobic cycle (Gardner, 1991). In the course of this aerobic reaction of LOX (Davies and Slater, 1987) only alkoxyl and acyl radicals have been detected by EPR.

Contrary, at anaerobic conditions lack of oxygen results in redox cycling of LOX by its substrate and hydroperoxide product with release of free pentadienyl 1 and alkoxyl radicals 2 (Gardner, 1991) (Scheme 2).

It was calculated that 1% of the linoleate radicals 1, that are formed after hydrogen abstraction, dissociate from the active site before enzymic oxygen insertion has occurred. Nevertheless, at aerobic conditions the number of these radicals that undergo enzymic oxygen insertion is two orders of magnitude larger than the number of free radicals that dissociate from the enzyme (Schilstra et al.,

Scheme 2. Anaerobic cycle: Formation of free pentadienyl 1 and alkoxyl radicals 2 generated by oxygen depletion.
1993). Oxygen depletion may enhance this free radical generation.

Further evidence for enhanced generation of free radicals at anaerobic conditions was obtained by Nelson et al.: they demonstrated that purple LOX-1, a 1:1 complex of LOX-1 with 13S-HPODE, favoured the formation of peroxyl radicals under conditions of O2 saturation; whereas depletion of O2 from purple LOX-1 led to detection of pentadienyl radicals 1 (Nelson et al., 1990; Nelson et al., 1994; Davies and Slater, 1987).

In conclusion, free radicals respectively secondary ROS generated to a higher extent at anaerobic conditions may damage the active site of LOX producing modified histidines, i.e., 2-oxo-histidine (Uchida and Kawakishi, 1994). These modified histidines have no iron binding capacity, which results in iron ion release from the active site (Ookawara et al., 1992; Sato et al., 1992).

As it is proposed that the existence of two cavities allows separate access of linoleic acid and dioxygen to the active site (Boyington et al., 1993) iron release caused by O2-depletion may be a means of substrate controlled regulation of LOX activity. In general, one may deduce that LOX activity is adapted to substrate (oxygen) supply by central iron release. These covalent modifications appear to “mark” a protein for subsequent proteolytic turnover (Rivett et al., 1985). Corresponding proteases have been purified from rodent liver (Rivett, 1985) and from E. coli (Oliver et al., 1994).

Our experiments demonstrate that the release of iron ions occurs in processes in which lipoxygenases are involved. Since lipoxygenases are liberated by cell damaging processes, in all these situations iron ions are released explaining the in vivo start of nonenzymic LPO. A decrease in oxygen supply is observed for instance in myocardial infarction (Dudda et al., 1996). As a consequence high increases of LPO products are observed. In LPO reactions, epoxides and α,β-unsaturated aldehydes are generated which are highly toxic and may contribute to the damaging effects observed after a myocardial infarction.

Acknowledgements

We thank Fonds der Chemischen Industrie for financial support of this work. We are also grateful to Mr. W. Kern for purification of solvents.


