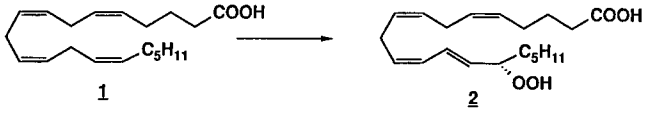
Tetrahedron Letters,Vol.27,No.31,pp 3585-3588,1986 0040-4039/86 $3.00 + .00
Printed in Great Britain Pergamon Journals Ltd.

A NEW IRREVERSIBLE INHIBITOR OF SOYBEAN LIPOXYGENASE; RELEVANCE TO MECHANISM

E. J. Corey, Marc d'Alarcao, and Seiichi P. T. Matsuda
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Summary: The mechanism of the oxidation of arachidonic acid by soybean lipoxygenase has been studied kinetically with the help of a newly designed inhibitor. It is proposed that olefin complexation with Fe(III) activates arachidonic acid for deprotonation by a basic group on the enzyme leading to an intermediate which then combines with dioxygen.

Enzymatic lipoxygenation of unsaturated fatty acids containing two *cis* double bonds attached to methylene is a process of considerable biological importance, for example, in the biosynthesis of prostaglandins and leukotrienes. This dioxygenation process is typified by the conversion of arachidonic acid (**1**) to the corresponding 15-(S)-hydroperoxide (**2**) by means of soybean lipoxygenase (SB-LO), the most intensively studied of the many known lipoxygenases. The related subjects of mechanism(s) of enzymatic lipoxygenation and rational design of specific LO inhibitors are currently of strong interest to investigators in a wide range of fields. Presently available evidence suggests that the catalytically active form of soybean



1 2

lipoxygenase contains high-spin d^5 Fe(III).[1] We recently suggested that Fe(III) probably functions to activate the fatty acid by complexation with the double bond undergoing oxygenation,[2] since high-spin Fe(III) is unlikely to interact effectively with O_2. Proton abstraction from the doubly allylic methylene group would then be facilitated either because of concerted electron transfer to Fe(III) (to form a pentadienyl radical) or covalent bonding to Fe at the terminal pentadienyl carbon.[3] Either possibility would set the stage for reaction with O_2 to form product. These arguments were derived in part from the study of 7-thiaarachidonic acid (**3**) which was found to be an irreversible inhibitor for the 5-LO from rat basophilic leukemic cells (RBL-1), in contrast to the corresponding sulfoxide which was only a competitive inhibitor.[2] This last observation also seems to weigh against mechanisms in which Fe(III) activates the enzyme for hydrogen atom abstraction by an

enzymic radical, for example, by the sequence: Fe(III) + RS^- -> Fe(II) + RS•; RS• + **1** -> RSH + (**1**-H)•, since the enzymic radical is expected to add to the CH=CH-SO unit of **3** sulfoxide as readily as to the CH=CH-S unit of **3**. In view of the highly informative results obtained from the study of **3** and the corresponding sulfoxide with the RBL-1 5-LO enzyme and the recent availability of 10-thia and 13-thiaarachidonic acids (**4** and **5**),[4] our research has been extended with the results presented herein on SB-LO. The new findings both parallel those from previous work[2] with the 5-LO from RBL-1 cells and lead to similar conclusions.

7-Thiaarachidonic acid **3** serves as a substrate for SB-LO under standard conditions (pH 9.2, 0.2 M sodium borate buffer at 22°) and is converted to the 15-hydroperoxide, UV max at 234 nm.[5] Similarly, 10-thiaarachidonic (**4**) is cleanly transformed by SB-LO to the 15-hydroperoxide which displays characteristic UV absorption at 286 nm due to the chromophore S-CH=CH-CH=CH. In contrast, 13-thiaarachidonic acid (**5**) is not a substrate for SB-LO but is instead an irreversible inhibitor of the lipoxygenation of arachidonic acid. Kinetic studies of this inhibition revealed an O_2 and time dependence characteristic of enzyme induced self inactivation. Rates of enzyme inactivation were measured by incubating **5** at concentrations of 0 to 25 μM with SB-LO (0.7 μg/ml, 6 nM) at pH 9.2 and 22° in air for varying times (0-10 min), adding arachidonic acid and measuring the change in UV absorbance at 236 nm to determine initial velocity of the lipoxygenation of arachidonic acid.[6,7] An apparent rate constant was determined by plotting log (percent remaining activity) vs preincubation time. A double reciprocal plot of apparent *k* vs conc. of inactivator **5** were linear in the range 0.3-2.4 μM. The reciprocal of the y-intercept provided the rate constant for inactivation, *ki* = 0.41 min^{-1} and the negative reciprocal of the x-intercept gave the binding constant for **5**, *Ki* = 0.8 μM. At 1 atm of O_2 the rate of inactivation was approximately double the rate in air, whereas under an atmosphere of argon, inactivation was barely observable at 10 min. Activity of SB-LO inhibited by incubation with **5** could not be restored by dialysis. The *Ki* value of 0.8 μM indicates that **5** is more tightly bound to SB-LO than is arachidonic acid (*Km* = 13 μM).[8,9]

It is known that SB-LO is capable of converting arachidonic acid in the presence of sodium borohydride and oxygen at pH 9.2 (standard conditions above) to the 15-alcohol corresponding to **2** (15-HETE).[10] Evidently, the powerful reducing agent borohydride cannot penetrate into the catalytic Fe(III) unit of SB-LO and functions merely as a reducing agent for hydroperoxide product **2**. We have observed that 13-thiaarachidonic acid (**5**) inactivates SB-LO under aerobic conditions even in the presence of sodium borohydride. Rates of inactivation

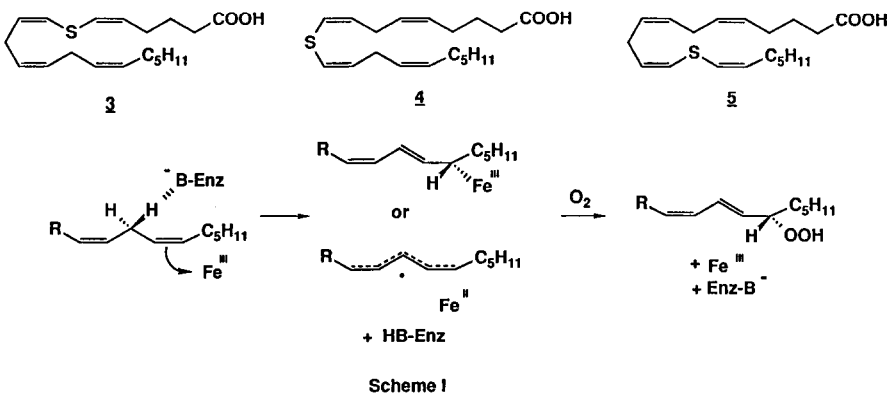
of SB-LO by the combination of **5** and O_2 are similar in the presence or absence of borohydride, consistent with the view that the enzyme is unaffected by this reagent.

The sulfoxide of **5** was prepared by oxidation in 3:2 methanol-water solution with 1.5 equiv.of sodium periodate at 23° for 7 hr (UV max < 208 nm, R_f 0.42 vs 0.53 for **5** on silica plates with 9:1 methylene chloride-methanol for elution). The sulfoxide of **5** was not a substrate for SB-LO and did not cause irreversible inactivation of SB-LO, but behaved simply as a competitive inhibitor, K_i = 66 μM.

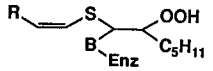
These findings with **5** and the corresponding sulfoxide and SB-LO parallel our earlier results[2] with 7-thiaarachidonate (**3**) and its sulfoxide as inhibitors of the 5-lipoxygenase from RBL-1 cells.

The data on inhibition of SB-LO by **5** and its sulfoxide are most simply understood in terms of the mechanistic formulation outlined in Scheme I. Fe(III) is thought to function as either a Lewis acid or electron acceptor by virtue of a vacant coordination site in proximity to the 14,15-double bond of substrate **1** with the result that deprotonation of the 13-methylene group by an enzymic base is facilitated. The intermediate organoiron compound or radical thus formed is oxygenated subsequently. This mechanism leads to the expectation that 13-thiaarachidomic acid could deactivate SB-LO by attaching covalently to the activated site as shown in **6**. The inability of the sulfoxide of **5** to inactivate SB-LO is consistent with the reduced availability of electrons in the 14,15-double bond of the sulfoxide. It also argues strongly against a mechanism in which the abstraction of hydrogen from C(13) is effected by an enzymic radical (e.g., RS• formed from RS^- and Fe(III))since such a radical could equally well add to the 14,15-double bond of **5** or its sulfoxide.

The fact that thia acids **3** and **4** serve as substrates and not inactivators of SB-LO is also consistent with the mechanism shown in Scheme I. The values of *K*m and Vmax were found to be 2.4 μM and 49 sec^{-1} for **3**, 10 μM and 232 sec^{-1} for **4**, and 13 μM and 197 sec^{-1} for **1**.[11]



Scheme I



6

REFERENCES AND NOTES

1. (a) J.J.M.C. DeGroot, G.A. Veldink, J.F.G. Vliegenthart, J. Boldingh, R. Wever, and B.F. van Gelder, Biochem. Biophys. Acta, **377**, 71 (1975); (b) S. Slappendel, B.G. Malström, L. Petersson, A. Ehrenberg, G.A. Veldink, and J.F.G. Vliegenthart, Biochem. Biophys. Res. Commun., **108**, 673 (1982); (c) J.E. Grunwald, M.S. Alexander, R. H. Fertel, C.A. Beach, L.K. Wang and J.R. Bianchine, ibid. **96**, 817 (1980); (d) E.K. Pistorius, B. Axelrod, and G. Palmer, J. Biol. Chem., **251**, 7144 (1976); (e) R.H. Cheesbrough and B. Axelrod, Biochem, **22**, 3837 (1983)

2. E.J. Corey, J.R. Cashman, T.M. Eckrich, and D.R. Corey, J. Am. Chem. Soc., **107**, 713 (1985).

3. Despite the fact that electron spin resonance studies of the interaction of polyunsaturated fatty acids with SB-LO at low oxygen concentration reveal the presence of carbon free radicals,[1a] it is not certain at present whether such radicals are obligatory intermediates.

4. E.J. Corey, M. d'Alarcao, and K.S. Kyler, Tetrahedron Letters, **26**, 3919 (1985).

5. Lipoxygenase products were reduced to the corresponding alcohols and esterified (CH_2N_2) for characterization. Satisfactory high-field pmr and mass spectral data were obtained for new compounds described herein.

6. Incubations were conducted using 3 ml of buffer adding **5** in 10 µl of ethanol, then adding enzyme, and finally after an appropriate time period adding 6 µl of 5 mM arachidonic acid in ethanol.

7. The oxidation of arachidonic acid by SB-LO showed the well known induction period (lag time) of several seconds due to the requirement for substrate-induced oxidation of the native (Fe(II)) form of SB-LO to the catalytically active Fe(III) form. The initial velocity of the lipoxygenation of arachidonic acid was measured after this induction period but before 2 min as the largest slope of the plot of absorbance at 236 nm vs time.

8. It has been observed that the inactivation of SB-LO by **5** under standard aerobic conditions does not proceed to completion. This effect is more noticeable as enzyme concentration is increased and, in addition, the rate of inactivation of enzyme decreases with increasing SB-LO concentration. These observations suggest a catalytically active aggregate of SB-LO which is resistant to aerobic inactivation by **5**.

9. The lag time for oxidation of **1** by SB-LO is increased in the presence of increasing concentrations of the 13-thia acid **5**. This fact indicates that **5** binds competitively to the native Fe(II) form of SB-LO in such a way as to retard conversion to the catalytically active Fe(III) form.

10. J.E. Baldwin, D.I. Davies, L. Hughes, and N.J.A. Gutteridge, J. Chem. Soc. Perkin I, 115 (1979).

11. This research was supported by the National Institutes of Health.

(Received in USA 22 May 1986)