The Action of Soybean Lipoxygenase-1 on 12-Iodo-*cis*-9-octadecenoic Acid: The Importance of C(11)−H Bond Breaking†

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ABSTRACT: Previous work has demonstrated that the ferric form of soybean lipoxygenase-1 will catalyze an elimination reaction on 12-iodo-*cis*-9-octadecenoic acid (12-IODE) to produce 9,11-octadecadienoic acid and iodide ion. Elimination is accompanied by irreversible inactivation of the enzyme on 1 out of 10 turnovers. In the present work, 11,11-dideuterio-12-IODE (D_2-12-IODE) was synthesized and used to demonstrate that both the elimination reaction and inactivation of the enzyme exhibit very large kinetic isotope effects. The rates with the deuterated compound are so low that the isotope effects are difficult to quantify, but they appear to be comparable to the isotope effects previously observed for the normal reaction catalyzed by lipoxygenase and much larger than can be explained by zero-point energy considerations. ESR spectroscopy was used to demonstrate that 12-IODE can reduce ferric lipoxygenase to the ferrous form, and a large isotope effect on this process was observed with D_2-12-IODE. It is proposed that the pathway leading to reduction and inactivation by 12-IODE is initiated by homolytic cleavage of the C(11)−H bond. Elimination could be initiated either by homolytic or by heterolytic cleavage of this bond. The results suggest that very large isotope effects may be a general feature of C−H bond cleavages catalyzed by this enzyme.

Lipoxygenases are non-heme iron proteins that catalyze the oxygenation of 1,4-dienes to produce conjugated diene hydroperoxides (*1*, *2*). Mammalian lipoxygenases (*3*) catalyze key steps in the conversion of arachidonic acid into mediators of inflammation and anaphylaxis (*4*), and may be involved in the development of atherosclerotic lesions (*5*) and in tumor metastasis (*6*). Plant lipoxygenases are involved in the synthesis of plant growth regulators and other substances that have been implicated in development, wound healing, defense against pathogens, and other functions (*7*−*10*).

Much of our structural and mechanistic insight into lipoxygenases comes from studies on soybean lipoxygenase-1, which acts on polyunsaturated fatty acids in which a 1,4-diene unit begins on the sixth carbon from the methyl terminus (*7*, *8*, *11*). For example, linoleic acid is converted to 13-hydroperoxyoctadecadienoic acid (13-HPOD),[1] as shown in Scheme 1. In freshly purified lipoxygenase-1, most of the iron is in the Fe(II) state, which can be oxidized to the catalytically active Fe(III) state by 13-HPOD (*11*−*14*). The crystal structure of the Fe(II) form of the enzyme has been determined (*15*, *16*).

There is considerable evidence that catalysis by soybean lipoxygenase-1 is initiated by reaction of the ferric form of the enzyme with linoleic acid to generate an intermediate, which reacts with oxygen (*11*, *17*−*19*). It has been proposed (*11*, *17*) that this intermediate is a pentadienyl radical bound to the ferrous enzyme; such an intermediate might be generated by transfer of a hydrogen atom from the substrate to a ferric hydroxide moiety (*20*, *21*), as shown in Scheme 2. The exact nature of the intermediate is not certain, however; other possible candidates include an organoiron species (*22*) and a Δ^{12}-[9,10,11]-allyl radical bound to the ferrous enzyme (*23*).

Studies on 11,11-dideuteriolinoleic acid (*24*−*27*) and, more recently, on 11-deuteriolinoleic acid (*28*) demonstrate that the reaction catalyzed by lipoxygenase-1 exhibits a primary kinetic isotope effect that is much larger than can be explained by zero-point energy considerations. Observed isotope effects range from 20 to 80, and the intrinsic isotope effect on the C−H bond breaking step is thought to be about 80 at 30 °C (*26*, *28*). The large isotope effect is not due to a magnetic field effect (*25*) and is probably not due to branching (*26*). The magnitude and temperature dependence

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[1] Abbreviations: 13-HPOD, 13-hydroperoxy-9,11-octadecadienoic acid; 12-IODE, 12-iodo-*cis*-9-octadecenoic acid; D_2-12-IODE, 11,11-dideuterio-12-iodo-*cis*-9-octadecenoic acid; 12-BrODE, 12-bromo-*cis*-9-octadecenoic acid; 9,11-ODA, 9,11-octadecadienoic acid; THF, tetrahydrofuran; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; ESR, electron spin resonance; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; CI-MS, chemical ionization mass spectrum; EI-MS, electron impact mass spectrum; GC, gas chromatography; HPLC, high-performance liquid chromatography; UV, ultraviolet.

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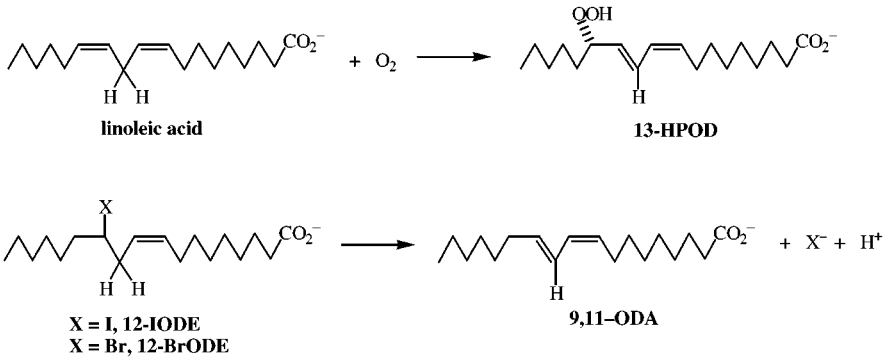
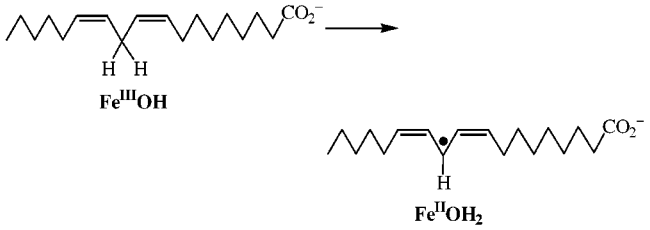
Scheme 1



Scheme 2



of this isotope effect have led to the conclusion that C−H bond breaking involves hydrogen tunneling (*27*−*29*), and possible models have been explored theoretically (*30*, *31*). A large isotope effect ($^{D}[k_{cat}/K_m] = 47$) has recently been reported for a mammalian lipoxygenase (*32*). A large isotope effect, estimated to be between 50 and 100, has also been observed for the reaction catalyzed by methane monooxygenase from *Methylosinus trichosporium* (*33*).

To probe the mechanism by which soybean lipoxygenase catalyzes C−H bond cleavage, we have been investigating the action of lipoxygenase on analogues of linoleic acid in which the C(12)−C(13) double bond is replaced by a potential leaving group at C(12) (Scheme 1). Our initial studies led to the discovery that 12-iodo-*cis*-9-octadecenoic acid (12-IODE) is a time-dependent, irreversible inactivator of ferric lipoxygenase (*34*). Subsequent work (*35*) demonstrated that ferric lipoxygenase catalyzes elimination reactions on both 12-IODE and 12-BrODE to produce 9,11-octadecadienoic acid (9,11-ODA) and halide ion (see Scheme 1). In the case of 12-IODE, about 10 turnovers of elimination occur, on average, before the enzyme becomes inactivated. No inactivation can be detected with 12-BrODE. The presence of free radical scavengers, such as ascorbate, protects the enzyme from inactivation by 12-IODE but does not affect the elimination reaction (*35*). This result implies that the pathway leading to inactivation involves scavengeable radicals. Our working hypothesis is that inactivation occurs by a pathway in which 12-IODE transfers one electron to ferric lipoxygenase to produce ferrous lipoxygenase plus a radical derived from 12-IODE. The relationship between this pathway and the pathway leading to elimination is not clear from our previous work.

The work reported in this paper had three objectives. The first was to determine whether C−H bond breaking is involved in the pathway leading to inactivation by 12-IODE. To answer this question, 11,11-dideuterio-12-IODE (D_2-12-IODE) was synthesized and used to test for a kinetic isotope effect on inactivation. The second objective was to use D_2-12-IODE to determine whether the elimination reaction catalyzed by lipoxygenase exhibits a very large isotope effect, comparable to that in the oxygenation reaction catalyzed by lipoxygenase. The third objective was to use ESR spectroscopy to test the hypothesis that 12-IODE reduces ferric lipoxygenase to the ferrous form and to use D_2-12-IODE to determine whether C(11)−H bond breaking is involved in this process.

MATERIALS AND METHODS

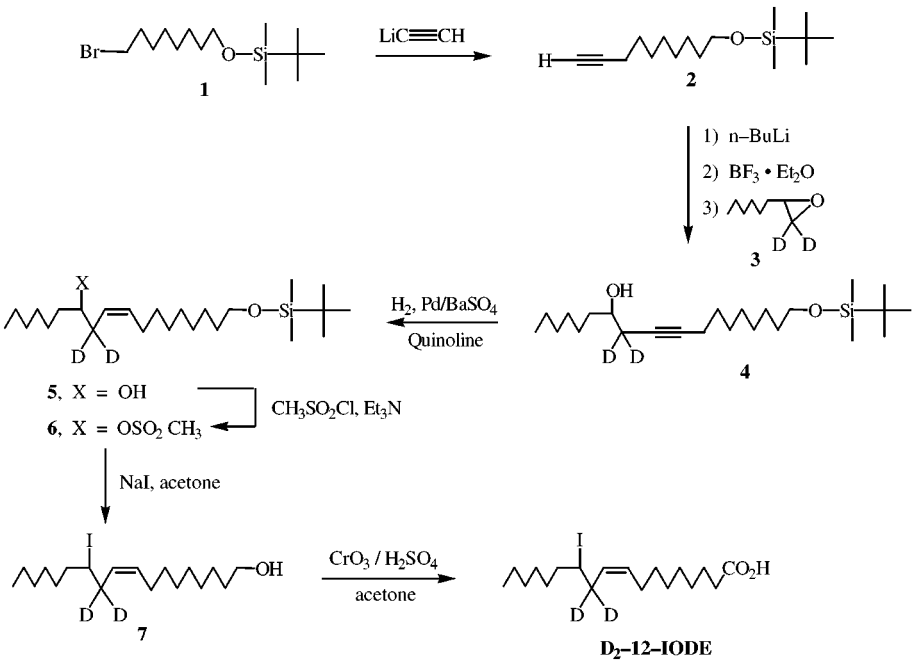
Materials. Soybean lipoxygenase-1 was purified by the method of Axelrod (*36*), and its concentration was determined spectrophotometrically using $A^{0.1\%}_{280nm} = 1.6$ (*37*). Unless otherwise noted, reagent chemicals were obtained from Aldrich or Sigma and used as supplied.

General Synthetic Methods. Anhydrous THF and ether were distilled under nitrogen from sodium diphenylketyl. Organic extracts were dried with $MgSO_4$ prior to concentration. Flash chromatography (*38*) was carried out using silica gel 60 (230−400 mesh, Aldrich). NMR data were obtained on a Bruker 300 MHz spectrometer, and chemical shifts are reported relative to tetramethylsilane. GC/MS analyses were carried out on an Extrel ELQ 400 system.

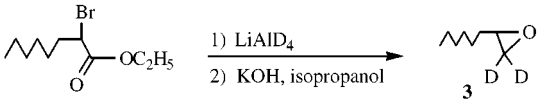
Synthesis of D_2-12-IODE. This substance was synthesized by the method outlined in Scheme 3. The key step was the reaction of the lithium acetylide derived from **2** with 1,1-dideuterioepoxyoctane (**3**) in the presence of BF_3−etherate (*39*) to produce **4**. The required deuterated epoxide, **3**, was prepared by reduction of ethyl 2-bromooctanoate with $LiAlD_4$ followed by cyclization of the resulting deuterated alcohol under basic conditions (Scheme 4). The triple bond in **4** was reduced to a cis double bond by catalytic hydrogenation, and the hydroxyl group was converted to a methanesulfonate to produce **6**. Treatment of **6** with sodium iodide in refluxing acetone converted the methanesulfonate to an iodide and also removed the *tert*-butyldimethylsilyl protecting group to give **7**, which was oxidized to D_2-12-IODE using Jones reagent (*40*). A sample of undeuterated 12-IODE was synthesized by this route using undeuterated 1,2-epoxyoctane, and the product of this synthesis had spectroscopic properties identical to those of samples of 12-IODE prepared by our previously published method (*34*). The experimental procedures for the synthesis of the deuterated material are presented below.

8-(Dimethyl-tert-butylsilyloxy)-1-bromooctane (***1***). A solution of 8-bromo-1-octanol (4.75 g, 22.8 mmol), *tert*-butyldimethylsilyl chloride (4.02 g, 26.6 mmol), and imidazole

Scheme 3



Scheme 4



(3.71 g, 54.4 mmol) in dimethylformamide (10 mL) was stirred at room temperature for 10 h (*41*). GC/MS analysis indicated that protection of the hydroxyl group was nearly complete at this point and that longer reaction times led to significant displacement of bromide in **1** by chloride. The reaction was quenched by addition of H_2O (200 mL) and extracted with hexanes (3 × 100 mL). Evaporation of the hexanes and fractionation of the residue by flash chromatography (hexanes/ethyl acetate, 15:1) yielded 5.9 g (80%) of **1** as a clear oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.56 (t, J = 6.4 Hz, 2 H, CH_2O), 3.36 (t, J = 6.8 Hz, 2 H, CH_2Br), 1.82 (qi, J = 6.8 Hz, 2 H), 1.15−1.6 (m, 10 Hz), 0.86 (s, 9 H, $C(CH_3)_3$), 0.01 (s, 6 H, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 63.2 (CH_2O), 33.8 (CH_2Br), 32.8, 32.8, 29.2, 28.8, 28.1, 26.0, 25.7, 18.3, −5.3; EI-MS: *m/e* 324/322 (M^+), 267/265 (M^+ − *t*-Bu).

10-(Dimethyl-tert-butylsilyloxy)-1-decyne (***2***). Compound **1** (3.78 g, 11.7 mmol) was added dropwise under N_2 over 1 h to a stirred solution of 1.23 g (13.3 mmol) of lithium acetylide−ethylenediamine complex (Aldrich) in 6 mL of dimethyl sulfoxide at 8 °C (external bath temperature) (*42*). When the addition was complete, the mixture was stirred for 2 h at room temperature. The reaction was quenched by addition of 50 mL of H_2O and then extracted 3 times with hexanes. Evaporation of the hexanes followed by flash chromatography (hexanes/ethyl acetate, 15:1) gave 2.5 g (80%) of **2** as a clear oil. ^{1}H NMR ($CDCl_3$) δ 3. 51 (t, J = 6.4 Hz, 2 H, CH_2O), 2.09 (dt, J = 2.6, 6.9 Hz, 2 H, $CH_2C{\equiv}C$), 1.84 (t, J = 2.6 Hz, 1 H, $HC{\equiv}C$), 1.15−1.55 (m, 12 H), 0.82 (s, 9 H, $C(CH_3)_3$), −0.04 (s, 6 H, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ 84.6, 68.0, 63.2, 32.8, 29.3, 29.1, 28.7, 28.5, 26.8, 25.9, 25.7, 18.4, −5.3; EI-MS: *m/z* 268 (M^+), 211 (M^+ − *t*-Bu).

1,1-Dideuterio-1,2-epoxyoctane (***3***). Solid $LiAlD_4$ (0.5 g, 11.9 mmol) was added in 15 portions at 1 min intervals to a stirred solution of 6.0 g (24 mmol) of ethyl 2-bromooctanoate (Lancaster) in 50 mL of anhydrous ether at 0 °C under N_2. The mixture was stirred for 15 min at 0 °C and for 3 h at room temperature. Subsequent addition of 0.5 mL of H_2O followed by 0.5 mL of 15% NaOH and 1.5 mL of H_2O (*43*) produced a granular precipitate, which was removed by filtration. The filtrate was partitioned between 30 mL of CH_2Cl_2 and 30 mL of H_2O, and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic phases were concentrated on a rotary evaporator to an oil, which was treated with a solution of 1.75 g of KOH in 40 mL of methanol. A white precipitate formed almost immediately. The mixture was stirred for 30 min at room temperature and then partitioned between ether (100 mL) and H_2O (100 mL). The aqueous layer was washed with ether (2 × 50 mL), and the combined ether extracts were concentrated on a rotary evaporator. The cloudy residual liquid was suspended in H_2O (15 mL) and then extracted with CH_2Cl_2 (2 × 15 mL). The CH_2Cl_2 extracts were concentrated to a clear liquid that was distilled under reduced pressure to give 1.6 g (52%) of **3** at 74−76 °C (22 mm). ^{1}H NMR ($CDCl_3$) δ 2.90 (t, J = 4.5 Hz, 1 H), 1.3−1.6 (m, 10 H), 0.89 (t, J = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 52.1, 34.5, 31.8, 29.1, 26.0, 22.6, 14.0; CI-MS(CH_4): *m/z* 131 (M + 1).

11,11-Dideuterio-12-hydroxy-1-(dimethyl-tert-butylsilyloxy)-9-octadecyne (***4***). *n*-Butyllithium (1.3 mL of a 1.6 M solution in hexanes, 2.1 mmol) was added by syringe to a stirred solution of **2** (0.54 g, 2.0 mmol) in 2.5 mL of dry THF and maintained at −78 °C under N_2. Boron trifluoride diethyl etherate (0.30 mL, 2.4 mmol, distilled before use) was subsequently added, and the resulting mixture was stirred for 15 min at −78 °C. Finally, a solution of **3** (0.14 g, 1.1 mmol) in dry THF (2.0 mL) was added. The reaction mixture was stirred at −78 °C for 30 min and subsequently warmed to room temperature and stirred for an additional 15 min.

The reaction was quenched by addition of a solution of ammonium chloride (0.11 g) in H_2O (20 mL) and extracted with hexanes (3 × 20 mL). Evaporation of the hexanes followed by flash chromatography (hexanes/ethyl acetate, 15:1) gave 0.57 g (72%) of **4** as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 3.65 (m, 1 H, C*H*OH), 3.57 (t, J = 7 Hz, 2 H, CH_2OSi), 2.13 (t, J = 7 Hz, 2 H, $CH_2C{\equiv}C$), 1.2−1.5 (m, 22 H), 0.89 (s, 9 H, $C(CH_3)_3$), 0.88 (t, 3 H, CH_3), 0.02 (s, 6 H, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ 83.3, 76.2, 70.2, 63.4, 36.3, 32.9, 31.9, 29.4, 29.4, 29.2, 29.1, 28.9, 26.1, 25.9, 25.7, 22.7, 18.8, 18.5, 14.2, −5.2.

11,11-Dideuterio-12-hydroxy-1-(dimethyl-tert-butylsilyloxy)-cis-9-octadecene (***5***). Compound **4** (120 mg) was stirred with $Pd/BaSO_4$ (6 mg) and quinoline (3 μL) in methanol (3 mL) under hydrogen for 40 min and then diluted with methanol (1 mL) and centrifuged. The supernatant was concentrated to give 80 mg of **5**, which was homogeneous by GC. In some runs, a longer reaction time was required (as judged by GC) to completely reduce **4** to **5**. ^{1}H NMR ($CDCl_3$) δ 5.5 (m, 1 H, $CH_2CH{=}CH$), 5.34 (d, J = 11 Hz, $CD_2CH{=}CH$), 3.55 (m, 1 H, C*H*OH), 3.55 (t, J = 6.7 Hz, 2H, CH_2OSi), 2.0 (m, 2 H, $CH_2C{=}C$), 1.2−1.7 (m, 22 H), 0.85 (s, 9 H, $C(CH_3)_3$), 0.85 (t, 3 H, CH_3), 0.02 (s, 6 H, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ 133.5, 125.2, 71.4, 63.4, 36.9, 32.9, 31.9, 29.8, 29.6, 29.5, 29.5, 29.3, 27.5, 26.1, 25.9, 25.8, 22.7, 18.4, 14.2, −5.2; CI-MS(isobutane): m/z 401 (M + 1).

11,11-Dideuterio-12-iodo-cis-9-octadecenol (***7***). The general method of Crossland and Servis (*44*) was used to convert **5** to its methanesulfonate, **6**. A solution of **6** (190 mg, 0.40 mmol) in 10 mL of 0.4 M NaI in acetone was refluxed for 6 h under nitrogen. The reaction mixture was then partitioned between 25 mL of 1 M $NaHSO_3$ and 25 mL of ethyl acetate. The organic layer was washed with 25 mL of 1 M NaCl and concentrated to give an oil (80 mg). ^{1}H NMR ($CDCl_3$) δ 5.5 (m, 1 H, $CH_2CH{=}CH$), 5.35 (d, J = 11 Hz, $CD_2CH{=}CH$), 4.05 (dd, J = 8.7, 4.5 Hz, 1 H, CHI), 3.61 (t, J = 7 Hz, CH_2O), 2.0 (m, $CH_2CH{=}CH$), 1.2−1.8 (m), 0.84 (t, CH_3). Signals for a small amount of unreacted methanesulfonate were also detectable. This substance was used without purification for the next step.

11,11-Dideuterio-12-iodo-9-cis-octadecenoic Acid (D_2-12-IODE). Compound **7** was oxidized to D_2-12-IODE in several portions. In a typical run, 25 mg of **7** in 1.5 mL of acetone was treated dropwise with Jones reagent (1.0 g of CrO_3 in 1 mL of H_2SO_4 diluted with 3 mL of H_2O) (*40*) until the orange color of the reagent persisted. The excess reagent was quenched by addition of several drops of 2-propanol, and the reaction mixture was partitioned between 3 mL of H_2O and 3 mL of ethyl acetate. The organic layer was washed twice with 1 M NaCl and concentrated to an oil, which was purified by HPLC in 1 mg portions using a 250 × 4.6 mm Alltech Adsorbosphere C18 (5 μm) column with acetonitrile/H_2O (85:15) as elution solvent (flow rate = 1.0 mL/min) to give 10 mg of purified D_2-12-IODE. This HPLC system is capable of separating 12-IODE from *trans*-12-IODE (*34*), and analytical HPLC of the purified material confirmed that it contained no detectable *trans*-12-IODE (limit of detection <2%).

The structure of D_2-12-IODE was confirmed by comparison of its spectroscopic properties with those of the unlabeled compound (*34*). The ^{1}H NMR spectrum of 12-IODE exhibits a multiplet at 2.65 ppm, assigned to the hydrogens on C(11), and this signal is absent from the spectrum of D_2-12-IODE. As expected, the multiplet at 5.38 ppm [due to the vinyl proton at C(10)] in 12-IODE is simplified to a doublet in the spectrum of the deuterated substance, and the coupling constant of this doublet (J = 11 Hz) confirms the cis stereochemistry. The multiplet at 4.1 ppm in 12-IODE [due to the hydrogen on C(12)] is simplified to a doublet of doublets in D_2-12-IODE. The remaining signals are identical in the spectra of the deuterated and undeuterated materials. The ^{13}C NMR spectrum of D_2-12-IODE is identical to that of the undeuterated material, except that it lacks the peak at 36.8 ppm, which we assign to carbon 11. Complete NMR data for D_2-12-IODE are as follows: ^{1}H NMR ($CDCl_3$) δ 5.5 (m, 1 H, $CH_2CH{=}CH$), 5.37 (d, J = 11 Hz, 1 H, $CD_2CH{=}CH$), 4.07 (dd, J = 8.7, 4.5 Hz, 1 H, CHI), 2.35 (t, J = 7.5 Hz, 2 H, CH_2CO_2H), 2.0 (m, 2 H, $CH_2CH{=}CH$), 1.2−1.8 (m, 10 H), 0.84 (t, J = 6.4, 3 H, CH_3); ^{13}C NMR-($CDCl_3$) δ 132.5, 127.3, 39.8, 38.4, 34.0, 31.7, 29.6, 29.4, 29.2, 29.2, 29.1, 28.5, 27.7, 24.7, 22.7, 14.1. The FTIR spectra of the deuterated and undeuterated samples were virtually identical, and both lacked the strong band at 960 cm^{-1} that is present in *trans*-12-IODE. Diazomethane was used to convert a small sample of D_2-12-IODE to its methyl ester, and a CI mass spectrum (CH_4) of this substance gave the expected M+1 peak at m/z 425. Ozonolysis of methyl D_2-12-IODE followed by treatment with dimethyl sulfide gave two products that were identical by capillary GC to the two products obtained from ozonolysis of unlabeled methyl 12-IODE.

12-(2-Naphthoxy)dodecanoic Acid. This substance was used as an internal standard for the HPLC assay for 9,11-ODA formation. 12-Bromododecanoic acid (Aldrich) was converted to its methyl ester by refluxing in methanol/H_2SO_4 (6:1) for 1 h. Reaction of methyl 12-bromooctanoate with 2-naphthol (1.25 equiv) and DBU (1.25 equiv) in dimethylformamide for 96 h at room temperature yielded methyl 12-(2-naphthoxy)dodecanoate, which was saponified by stirring with THF/1.0 M LiOH (1:1) at room temperature for 24 h. Acidification and two recrystallizations (ethanol/H_2O, 1:1) gave 12-(2-naphthoxy)dodecanoic acid (mp 86−88 °C).

HPLC Assay for Formation of 9,11-ODA. At time zero, 6.0 μL of a 12.5 mM solution of 12-IODE or D_2-12-IODE in ethanol was added to 1.5 mL of a solution of lipoxygenase and 50 μM 13-HPOD in 50 mM borate, pH 9.0, at 20 °C. At the indicated times (Table 1), 450 μL aliquots were withdrawn and quenched by addition to an equal volume of ice-cold acetonitrile/methanol/H_3PO_4 (12.5:5:0.2, v/v) that contained 5.0 μM 12-(2-naphthoxy)dodecanoic acid as internal standard. Aliquots (250 μL) of the quenched solutions were analyzed by HPLC on a 250 × 4.6 mm Alltech Adsorbosphere C18 (5 μm) column at 1.0 mL/min. Mobile phase A was 0.1% acetic acid in H_2O, and mobile phase B was 0.1% acetic acid in acetonitrile. Elution was carried out as follows: 0−3 min, 60% B; 3−6 min, linear gradient from 60 to 85% B; 6−30 min, 85% B. Detection was by UV absorbance at 234 nm. Concentrations of 9,11-ODA were determined from the ratio of the area of the 9,-11-ODA peak (ca. 20 min) to that of the peak for the internal standard (ca. 16 min) using a standard plot obtained with solutions of 0−25 μM 9,11-ODA, and 5.0 μM internal standard.

Determination of 12-IODE by HPLC with Evaporative Light-Scattering Detection. Solutions of 25−50 μM 12-IODE in 50 mM borate, pH 9.0, were diluted with equal volumes of quench solution [acetonitrile/methanol/H_3PO_4 (12.5, 5.0, 0.2, v/v)] containing 150 μM oleic acid as internal standard. Aliquots (400 μL) of these diluted solutions were analyzed by HPLC on a 250 × 4.6 mm Alltech Adsorbosphere C18 (5 μm) column at 1.0 mL/min. Mobile phase A was 0.1% acetic acid in H_2O, and mobile phase B was 0.1% acetic acid in acetonitrile. Elution was carried out as follows: 0−3 min, 60% B; 3−6 min, linear gradient from 60 to 88% B; 6−25 min, 88% B. Detection was carried out with a Varex MKIII evaporative light-scattering detector (Alltech) with a nitrogen flow of 2.1 L/min and a drift tube temperature of 90 °C. Using solutions of known concentration, it was found that plots of 12-IODE concentration vs the ratio of the peak areas of 12-IODE (ca. 18 min) to oleic acid (ca. 20 min) were linear, provided the final concentration of 12-IODE was between 12 and 20 μM; significant deviations from linearity occurred outside this range.

In assays for the consumption of 12-IODE or D_2-12-IODE, incubation mixtures were quenched at the appropriate times with an equal volume of quench solution (see above), and, if necessary, the solutions were further diluted with equal volumes of quench solution and borate buffer to bring the final concentration of 12-IODE into the linear response range of the detector. HPLC analysis was carried out as above, and concentrations of 12-IODE were determined from the peak area ratios using a standard plot prepared on the same day. A 2 μm in-line prefilter (Alltech) was used to protect the column from the high concentrations of protein present in some of the assays; despite this precaution, repeated injections of samples containing high concentrations of protein resulted in clogging of the column frit and/or peak broadening. Column performance could usually be restored by replacing the frit and washing the column with organic solvents.

ESR Experiments. ESR spectra were obtained on a Micro Now 8300 ESR spectrometer. To prepare lipoxygenase for ESR experiments, solutions of purified enzyme were adjusted to pH 9.0 by repeated cycles of dilution with 50 mM borate, pH 9.0, followed by concentration in an Amicon ultrafiltration cell with a YM 30 membrane. Further concentration to about 50 mg/mL was carried out in a Centricon-30 (Amicon). An aliquot of the concentrated solution was subjected to fluorescence titration with 13-HPOD to determine the amount of 13-HPOD required to minimize the fluorescence intensity at 325 nm following excitation at 280 nm; this value corresponds to the amount of 13-HPOD required to oxidize all of the ferrous enzyme in a particular sample to the ferric form (*45*). Samples of ferric lipoxygenase-1 for ESR were prepared by treating samples of the ferrous enzyme with the appropriate amount of 13-HPOD, as determined from the fluorescence titrations.

RESULTS

Isotope Effect on Inactivation. The data in Figure 1 indicate that little or no inactivation occurred when lipoxygenase (0.5 μM) was incubated with 50 μM D_2-12-IODE at 20 °C, pH 9.0, in the presence of 50 μM 13-HPOD. Under these conditions, rapid inactivation was observed with undeuterated 12-IODE. Indistinguishable results were obtained with two samples of undeuterated 12-IODE, one synthesized as described previously (*34*) and the other prepared by the same route used to synthesize the deuterated sample (Scheme 3). These results indicate that there is a very large kinetic isotope effect on the inactivation of soybean lipoxygenase by 12-IODE.

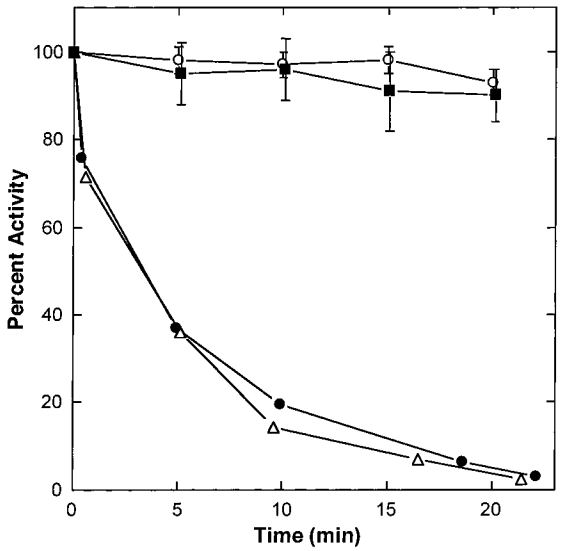


FIGURE 1: Time-dependent inactivation of soybean lipoxygenase by D_2-12-IODE and 12-IODE. Soybean lipoxygenase (0.5 μM) was incubated with (■) 50 μM D_2-12-IODE (average of four experiments), (○) no inactivator (average of two experiments), (●) 50 μM 12-IODE, synthesized as described previously (*34*), or (△) 50 μM 12-IODE, synthesized by the method in Scheme 3 starting with undeuterated 1,2-epoxyoctane. Each experiment was carried out at 20 °C in 50 mM borate, pH 9.0, containing 50 μM 13-HPOD and 0.8% ethanol. Time zero is the time of addition of the inactivator, dissolved in ethanol, or ethanol alone in the case of the control. At the indicated times, aliquots were withdrawn and assayed for lipoxygenase activity spectrophotometrically using linoleic acid as substrate (*34*). Error bars are standard deviations.

Table 1: Formation of 9,11-ODA from 12-IODE and D_2-12-IODE[a]

		yield of 9,11-ODA (μM)		
substrate (50 μM)	enzyme concn (μM)	10 min	20 min	30 min
12-IODE	5	26	23	
D_2-12-IODE	5	<1	1−2	
D_2-12-IODE	50	4		8

[a] Incubations were carried out at 20 °C in 50 mM borate, pH 9.0, in the presence of 50 μM 13-HPOD. At the indicated times, aliquots were withdrawn, quenched, and analyzed by HPLC with UV detection, as described under Materials and Methods.

Isotope Effect on Elimination. Having established that inactivation by D_2-12-IODE was insignificant, we set out to measure the isotope effect on the elimination reaction. In our initial experiments, samples of lipoxygenase (5 μM) were incubated with 50 μM 12-IODE or D_2-12-IODE at 20 °C, pH 9.0, in the presence of 50 μM 13-HPOD. After appropriate time intervals, aliquots were removed and analyzed for 9,11-ODA by HPLC, as described under Materials and Methods. Some of the results are presented in Table 1. With unlabeled 12-IODE, about half of the racemic substrate was converted to 9,11-ODA after 10 min. This result is consistent with essentially complete conversion of the active enantiomer over this time and is in good agreement with results obtained

Table 2: Consumption of 12-IODE or D_2-12-IODE in the Presence of Lipoxygenase[a]

substrate[b]	enzyme concn (μM)	time (min)	substrate[c] consumed (μM)
12-IODE	5	0.5	4.5 ± 0.7
12-IODE	5	1.0	9.2
12-IODE	5	5	22.5
D_2-12-IODE	5	20	<2
D_2-12-IODE	10	20	3.2
D_2-12-IODE	25	10	4.3 ± 0.4
D_2-12-IODE	25	20	6.5 ± 1.2
D_2-12-IODE	0	20	0.7 ± 0.2

[a] Incubations were carried out at 20 °C in 50 mM borate, pH 9.0, in the presence of 50 μM 13-HPOD. [b] Each experiment contained between 48.5 and 51.7 μM substrate; the exact initial concentration was determined by HPLC, as described under Materials and Methods. [c] The difference between the initial concentration of substrate and the concentration at the indicated time.

previously (*35*). The result in Table 1 was obtained using 12-IODE prepared by the route in Scheme 3; in other experiments, it was shown that the results are not significantly different with 12-IODE synthesized as described previously (*34*). In contrast, the formation of 9,11-ODA from D_2-12-IODE was barely detectable and difficult to quantify when 5 μM lipoxygenase was used. To observe a substantial amount of 9,11-ODA formation, the enzyme concentration had to be increased to 50 μM. Even at this concentration, only 8 μM 9,11-ODA was formed after 30 min.

The data in Table 1 indicate that there is a large isotope effect on the elimination reaction. The interpretation of the data is complicated, however, by the fact that 9,11-ODA breaks down slowly in the presence of high concentrations of lipoxygenase. This reaction has been noted previously (*35*) and is probably responsible for the fact that the yield of 9,-11-ODA produced from unlabeled 12-IODE in Table 1 is slightly lower at 20 min than at 10 min. The occurrence of this reaction has been confirmed by monitoring the consumption of authentic 9,11-ODA in the presence of lipoxygenase; the products of the reaction have not been identified. The reaction is sufficiently slow that it should not interfere with measuring the initial rate of formation of 9,11-ODA from unlabeled 12-IODE, but it probably does interfere with measuring the much slower initial rate at which 9,11-ODA is formed from D_2-12-IODE. Consequently, the data in Table 1 probably underestimate the rate of the elimination reaction with D_2-12-IODE, since a significant amount of the 9,11-ODA that is formed by this reaction is subsequently consumed.

The difficulty described in the previous paragraph could be avoided if the elimination reaction was monitored by disappearance of the substrate. Since 12-IODE absorbs only weakly in the UV, we developed an assay in which 12-IODE is detected by HPLC with an evaporative light-scattering detector (see Materials and Methods). The data in Table 2 were obtained using this assay. These results confirm that the isotope effect is very large. When 50 μM unlabeled 12-IODE was incubated with 5 μM lipoxygenase under the usual conditions (20 °C, pH 9.0, in the presence of 50 μM 13-HPOD), 12-IODE was consumed at an initial rate of 9.0 μM/min (based on the 0.5 min time point). With D_2-12-IODE, a concentration of 25 μM lipoxygenase was necessary in order

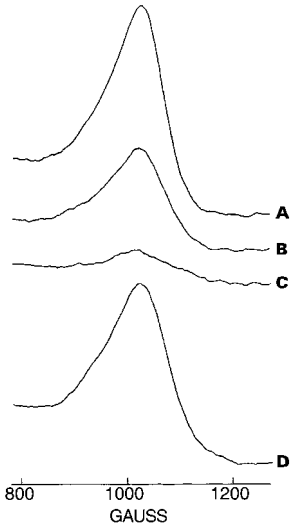


FIGURE 2: Effects of 12-IODE on the ESR spectrum of ferric lipoxygenase. (A) Ferric lipoxygenase (12 nmol). (B) Sample A plus 24 nmol of 12-IODE. (C) Sample B plus 48 nmol of 12-IODE. (D) Sample C plus 12 nmol of 13-HPOD. Each spectrum is the average of 6 scans obtained under the following conditions: spectrometer frequency, 9.0 GHz; modulation amplitude, 32 G; microwave power, 20 mW; temperature, 77 K.

to observe consumption of substrate at a quantifiable rate. Based on the 10 min time point (and correcting for the very slow nonenzymatic breakdown of 12-IODE), we estimate the initial rate to be about 0.43 μM/min at 25 μM lipoxygenase, which leads to an estimated rate of 0.086 μM/min at 5 μM lipoxygenase. These data are consistent with an isotope effect on the order of 100.

As expected from the results in Figure 1, spectrophotometric assays with linoleic acid indicate that no significant inactivation by D_2-12-IODE occurred under the conditions of the experiments in Table 2. This result demonstrates that the large isotope effect observed on elimination is not due to inactivation of the enzyme.

Reduction of Ferric Lipoxygenase by 12-IODE. ESR experiments were carried out to test the hypothesis that ferric lipoxygenase can be reduced by 12-IODE. The ferric form of lipoxygenase exhibits an ESR signal near $g = 6$; the ferrous form is ESR-silent. Spectrum A in Figure 2 shows the $g = 6$ region of the ESR spectrum at 77 K of 12 nmol of ferric lipoxygenase in 150 μL of 50 mM borate, pH 9.0. This sample was prepared by treatment of 19.5 nmol of purified lipoxygenase with 12 nmol of 13-HPOD. After the spectrum was obtained, the sample was thawed, treated with 24 nmol of 12-IODE (in 1.0 μL of ethanol), incubated for 4 min at room temperature, and refrozen. This treatment reduced the $g = 6$ signal by about 50% (spectrum B). No other changes were observed between 500 and 4000 G. Longer incubation periods at room temperature following addition of 12-IODE did not cause any further reduction of the $g = 6$ signal. Subsequent addition of 48 nmol of 12-IODE nearly eliminated the signal (spectrum C). Further additions of 12-IODE did not eliminate the small residual signal in spectrum C. The signal for the ferric enzyme was restored by addition of 12 nmol of 13-HPOD (spectrum D). These results indicate that treatment of ferric lipoxygenase with excess 12-IODE reduces most, but not quite all, of the ferric ions in the enzyme preparation. The results of several similar experiments indicate that 4−5 equiv of 12-IODE is

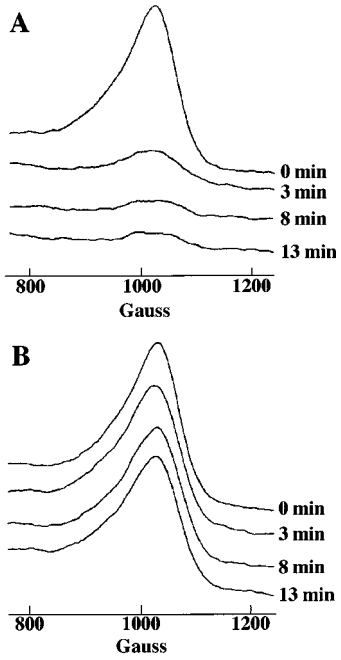


FIGURE 3: Effects of 12-IODE and D_2-12-IODE on the ESR spectrum of ferric lipoxygenase. Panel A: A solution of 80 μM ferric lipoxygenase was prepared in an ESR tube and frozen, and its ESR spectrum was recorded under the conditions of Figure 2 to give the 0 min scan. The sample was then thawed by immersion in a room-temperature water bath and treated with 12-IODE (final concentration = 500 μM) and incubated at 25 °C. At the indicated times, the sample was frozen and its ESR spectrum scanned. Panel B shows the spectra obtained in an identical experiment using D_2-12-IODE.

required to reduce the ESR signal to the level of the residual signal observed in Figure 2C.

It is possible that the reduction of the enzyme that was detected in Figure 2 might actually be due to the products of the elimination reaction. To test for this possibility, a control experiment was carried out in which 12 nmol of ferric lipoxygenase was incubated with 72 nmol of 9,11-ODA and 72 nmol of sodium iodide. The height of the $g = 6$ ESR signal was reduced to about 80% of the initial height (data not shown). Thus, some reduction in the intensity of the $g = 6$ signal can be observed in the presence of iodide and 9,11-ODA, but this is much less than what was observed after treatment with 12-IODE. This result demonstrates that most of the reduction detected in Figure 2 is due to 12-IODE itself rather than to the reduction of the ferric enzyme by the products of the elimination reaction.

Figure 3 shows the results of an experiment in which the reduction of ferric lipoxygenase (80 μM) by 12-IODE (panel A) and D_2-12-IODE (panel B) was monitored by ESR. The results indicate that the reduction of the ferric enzyme shows a large isotope effect.

DISCUSSION

Previous work implies that the pathway leading to inactivation by 12-IODE involves a radical (*35*). Formation of this radical might be triggered by a one-electron oxidation of the iodine or homolytic cleavage of the C(11)−H bond. The large isotope effect on inactivation provides compelling evidence for C(11)−H bond breaking. Since the normal reaction catalyzed by lipoxygenase on linoleic acid requires C−H bond breaking, the kinetic isotope effect reported here conclusively demonstrates that 12-IODE is a mechanism-based inactivator.

Our results indicate that there is a very large deuterium isotope effect on the lipoxygenase-catalyzed elimination reaction on 12-IODE. The reaction of the deuterated form is so slow that the isotope effect is difficult to quantify, but our data suggest it might be as high as 100 at 20 °C. It is clearly much larger than can be explained by zero-point energy considerations. Since D_2-12-IODE bears two deuterium atoms on carbon-11, it is not possible to separate the contributions of primary and secondary isotope effects to the large effect that we observe. In the case of the normal reaction, recent work on 11-deuteriolinoleic acid has demonstrated that the primary isotope effect is anomalously large and the secondary isotope effect is of normal magnitude (*28*). Presumably, this is also the case with D_2-12-IODE.

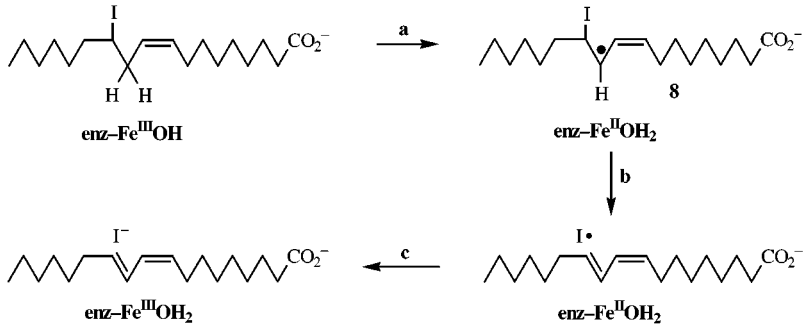
Large observed isotope effects can sometimes arise in branched pathways with more than one isotopically sensitive step (*46*). The processing of 12-IODE by lipoxygenase almost certainly involves branching, but the likelihood that branching is responsible for the large isotope effect is minimized by the fact that the effect was observed on consumption of substrate. The only sort of branched pathway that could account for a large isotope effect on substrate consumption is one in which an isotopically insensitive branch leads back to starting material (see appendix to ref *26*). As in the case of the normal reaction catalyzed by lipoxygenase, it is difficult to envision a chemically plausible mechanism that is consistent with this constraint.

Recent work has shown that the L546A mutant of soybean lipoxygenase oxygenates linoleic acid with an isotope effect that is essentially identical with that of the wild-type enzyme (*28*). Thus, at least in this one case, modification of the protein does not affect the special features of this enzyme that are responsible for the large isotope effect. Our results indicate that these special features remain operative when the substrate is modified to allow the energetically favorable dissociation of a leaving group.

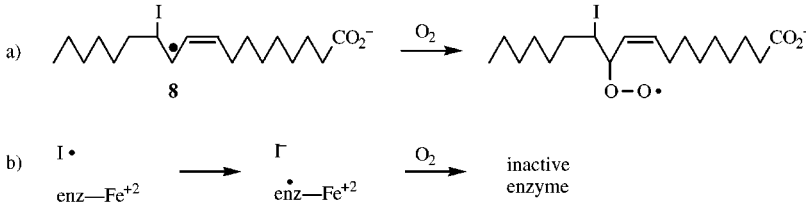
The ESR results demonstrate that 12-IODE can reduce ferric lipoxygenase to the ferrous form. The rate of reduction is much lower with D_2-12-IODE. This observation indicates that reduction, like inactivation, involves breaking of the C(11)−H bond in 12-IODE. The fact that the $g = 6$ signal due to ferric lipoxygenase is not completely eliminated by excess 12-IODE may be due to the presence of a small amount of catalytically inactive enzyme in our preparations. Since reduction by 12-IODE involves C−H bond breaking, it is likely that catalytically inactive enzyme would not be reduced. The residual signal can be eliminated by treatment with *N*-octylhydroxylamine, a reducing agent that does not require C−H bond breaking (*47*).

Presented in Scheme 5 is a mechanistic pathway that can explain both the elimination reaction and the reduction of the enzyme and also provide a starting point for explaining inactivation. According to this hypothesis, the processing of 12-IODE is initiated by homolytic cleavage of the C(11)−H bond by the ferric hydroxide moiety at the active site to produce a β-iodoallylic radical (**8**) and a hydrated ferrous ion. This step is analogous to the initial step in one of the proposed mechanisms for the oxygenation reaction catalyzed by lipoxygenase (Scheme 2). Homolytic fragmentation of **8**

Scheme 5



Scheme 6



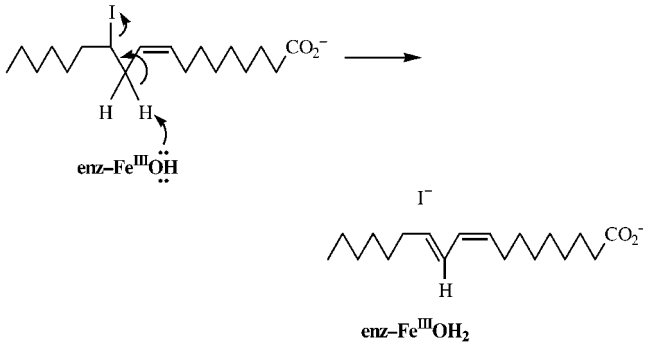
would produce 9,11-ODA and an iodine atom. If the iodine atom acquired an electron from the ferrous ion, the overall result would be an elimination reaction to convert 12-IODE to 9,11-ODA and iodide ion, which is the reaction that we observe. On some turnovers, the iodine atom might diffuse away from the active site and acquire an electron from some other source, possibly a residue on the surface of the protein. This would also lead to the observed products, I^- and 9,-11-ODA, but it would leave the iron in the ferrous state.

The mechanism in Scheme 5 is consistent with the fact that ferrous lipoxygenase does not catalyze an elimination reaction on 12-IODE (*35*). Furthermore, the hypothesis that iodine atoms sometimes diffuse away from the iron, leaving it in the ferrous form, leads to the prediction that the elimination reaction catalyzed by ferric lipoxygenase should cease after several turnovers unless excess 13-HPOD is present to reoxidize the ferrous enzyme to the ferric form. This is in accord with previous observations (*35*).

In the presence of excess 13-HPOD, lipoxygenase will convert about 50% of racemic 12-IODE to 9,11-ODA and iodide ion (*35*). This observation probably indicates that only one enantiomer of 12-IODE undergoes enzymatic elimination. The ESR results imply that 4−5 equiv of racemic 12-IODE is required to reduce the ferric enzyme; this presumably corresponds to 2−2.5 equiv of the active enantiomer. Within the context of Scheme 5, this stoichiometry implies that the iodine atom diffuses away from the iron on 40−50% of turnovers.

Earlier studies have demonstrated that 12-IODE inactivates the enzyme at a rate that allows for formation of about 10 iodide ions per molecule of enzyme inactivated (*35*). Inactivation requires O_2 (*34*), and the major pathway for inactivation does not involve covalent attachment of the carbon skeleton of the inactivator to the protein (*35*). A possible mechanism for inactivation is that on about 1 out of 10 turnovers, the β-iodoallylic radical, **8**, in Scheme 5 reacts with O_2 to form a peroxy radical (Scheme 6a), which then abstracts a hydrogen atom or an electron from a catalytically essential residue at the active site. Alternatively, it is possible that on 1 out of 10 turnovers, the iodine atom

Scheme 7



in Scheme 5 reacts with a functional group at the active site, which then reacts with O_2, leading to inactivation (Scheme 6b).

Steps a and b of Scheme 5 are analogous to a mechanism suggested by Wright and Nelson (*48*) to explain the reduction of ferric lipoxygenase by 12,13-epithiooleic acid. The observation of an isotope effect on the reduction of ferric lipoxygenase by D_2-12-IODE strengthens the case for the viability of this sort of mechanism. The loss of iodide atoms from β-iodoalkyl radicals is known to be rapid ($k > 5 \times 10^9$ s^{-1}) (*49*), and this process has been used to provide evidence for radical intermediates in photochemical dimerizations of pyrimidines (*50*) and in reactions of oligonucleotides with bleomycin (*51*).

An alternative mechanism for the elimination reaction is a heterolytic process in which the ferric hydroxide moiety accepts a proton, rather than a hydrogen atom, to trigger the loss of iodide ion and formation of a double bond (Scheme 7). It is conceivable that elimination takes place by this heterolytic mechanism while reduction occurs by a competing homolytic process—steps a and b of Scheme 5. According to this view, step c in Scheme 5 does not occur to a significant extent. An intriguing feature of this alternative hypothesis is the notion that ferric lipoxygenase can cleave a C−H bond either homolytically or heterolytically. This is plausible if the key player at the active site is a ferric hydroxide moiety. If this mechanistic duality occurs, our

results, combined with those of others *(24−28)*, imply that both homolytic and heterolytic C−H cleavages catalyzed by lipoxygenase exhibit anomalously large isotope effects. This possibility may not be as unlikely as it intuitively appears. In one of the models proposed to account for the isotope effect on the normal reaction (*30*), proton tunneling from the substrate to the ferric hydroxide moiety precedes electron transfer, but the two steps are inseparable, so that the process would be viewed as homolytic. If this idea is correct, there might also be a very large isotope effect on a heterolytic process, such as that in Scheme 7, since the proton tunneling step would still occur. Distinguishing between homolytic and heterolytic pathways for the elimination reaction is one of the goals of future work in our laboratory.

In sum, our results demonstrate that cleavage of the C(11)−H bond of 12-IODE is involved in elimination, reduction, and inactivation of the enzyme. Additional experiments to test the mechanistic ideas presented here are in progress.

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