Selenite Incubated with NADPH and Mammalian Thioredoxin Reductase Yields Selenide, Which Inhibits Lipoxygenase and Changes the Electron Spin Resonance Spectrum of the Active Site Iron†

Mikael Björnstedt,‡ Björn Odlander,§ Sergei Kuprin,‡ Hans-Erik Claesson,§ and Arne Holmgren*,‡

The Medical Nobel Institute for Biochemistry and Division of Physiological Chemistry 2, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

Received December 6, 1995; Revised Manuscript Received March 5, 1996⊗

ABSTRACT: Selenite and selenodiglutathione (GS-Se-SG) efficiently inhibited 5-lipoxygenase activity in sonicates of human monoclonal B-lymphocytes. The apparent IC_{50} of GS-Se-SG was 0.5 μM. The inhibitory effect of these compounds was observed within 10 min of incubation. In order to elucidate if the mechanism of inhibition by these compounds was result of direct interference with lipoxygenase or indirectly mediated by cellular factors, pure 15-lipoxygenase from soybeans was used as a model system for enzyme assays and electron spin resonance (ESR) measurements. Incubation of 15-lipoxygenase with a mixture of human placenta thioredoxin reductase (TR) or calf-thymus TR, selenite, and NADPH blocked the activity of the enzyme. Neither TR and NADPH nor selenite inhibited soybean lipoxygenase when incubated separately. These results suggest that selenite must be reduced to selenide in order to inhibit 5- and 15-lipoxygenase activities. Preincubation anaerobically of 15-lipoxygenase with chemically generated selenide (6 μM) resulted in a strong inhibition of activity, in assays with arachidonic acid in the presence of oxygen. In contrast, selenide exposed to air prior to preincubation did not inhibit the enzyme. Since selenide is known to be efficiently oxidized by oxygen and to form elemental selenium the results evidence that selenide was the inhibitor of lipoxygenase activity in the anaerobic preincubations. After incubation with TR, NADPH, and selenite or with chemically generated selenide, the ESR spectrum of 15-lipoxygenase changed: the dominant axial component with a peak at $g = 6.1$ decreased, and a rhombic form with a feature at $g = 4.28$ grew. The results suggest that selenide produced by the reduction of selenite reduces the active site iron to the ESR invisible state and changes the ligation geometry of the oxidized form.

The mammalian lipoxygenases are a family of structurally related enzymes, catalyzing the oxygenation of arachidonic acid (Yamamoto, 1992). This fatty acid is in cells of myeloid lineage and B-lymphocytes metabolized to leukotriene A_4 (LTA_4), a reaction catalyzed by 5-lipoxygenase (Samuelsson, 1983; Samuelsson & Funck, 1989; Jakobsson et al., 1991, 1992, 1995). LTA_4 can be further metabolized to either leukotriene B_4 (LTB_4) or leukotriene C_4 (LTC_4) (Samuelsson, 1983; Samuelsson & Funck, 1989). Several lines of evidence indicate that leukotrienes are involved in the pathophysiology of inflammatory diseases and asthma (Samuelsson, 1983; Kumlin et al., 1992). 15-Lipoxygenase has also been suggested to play a role in the development of atherosclerosis (Ylä-Herttuala et al., 1990). These two enzymes, 5-lipoxygenase and 15-lipoxygenase, share several common features regarding the mechanism of enzymatic activity. The active sites of these enzymes contain a non-heme binding iron that is essential for the catalytic activity (Yamamoto, 1992; Percival, 1991; Zhang et al. 1993; Chasteen et al., 1993; Zhang et al., 1995).

Selenium is an essential trace element. The best established function of selenium is its presence in the detoxifying enzyme glutathione peroxidase as a selenocysteine residue in the active site (Flohé, 1989). Selenium has been suggested to exert anti-inflammatory effects (Weitzel & Wendel, 1993), and patients with asthma were found to have decreased levels of selenium in plasma (Stone et al., 1989). Due to decreased peroxide tone, through induction of selenium-dependent glutathione peroxidases, selenium compounds have been considered to block the activity of lipoxygenases (Weitzel & Wendel, 1993). Inorganic selenium in the form of selenite is metabolized in mammalian tissues through stepwise reduction by thiols (Ganther, 1968, 1971; Kice et al., 1980; Kumar et al., 1992; Björnstedt et al., 1992, 1995). Glutathione is the most abundant thiol in mammalian cells, and selenodiglutathione (GS-Se-SG) is thus believed to be the major metabolite of inorganic selenium compounds in mammalian tissues (Hsieh & Ganther, 1975; Shamberger, 1985). Further reduction of GS-Se-SG by glutathione reductase (Ganther, 1971) or by the thioredoxin system (Björnstedt et al., 1992, 1995) will give rise to the highly reactive selenium anion, selenide. This anion is important for the formation of selenoamino acids (Böck et al., 1991)

† This investigation was supported by grants from the Karolinska Institute, the Swedish Cancer Society (961), the Swedish Medical Research Council (13X-3529 and 03X-7135), King Gustav V's 80-Year Fund, Magnus Bergvall's Stiftelse, and Knut och Alice Wallenberg's Stiftelse. S.K. was supported by grants from the Royal Swedish Academy of Sciences, Carl Trygger's Stiftelse, and Wenner-Grenska Samfundet. Support to S.K. from Sigurd and Elsa Goljes Memory Foundation is gratefully acknowledged.

* To whom correspondence should be addressed. Tel: +46-8-728 76 86. FAX: +46-8-728 47 16.

‡ The Medical Nobel Institute for Biochemistry.

§ Division of Physiological Chemistry 2.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1996.

S0006-2960(95)02876-5 CCC: $12.00

and thus for the formation of selenium-dependent glutathione peroxidases.

Thioredoxin (Trx)[1] is a 12 kDa ubiquitous protein with a redox active dithiol−disulfide in the active site (Holmgren, 1985). It operates together with TR and NADPH, the Trx-system, as a general protein disulfide reducing system (Holmgren, 1985):

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \overset{\text{TR}}{\rightleftharpoons} \text{Trx-(SH)}_2 + \text{NADP}^+$$

$$\text{Trx-(SH)}_2 + \text{protein-S}_2 \rightleftharpoons \text{Trx-S}_2 + \text{protein-(SH)}_2$$

One function for this system is to serve as a hydrogen donor to the essential enzyme ribonucleotide reductase (Thelander & Reichard, 1979). Thioredoxin can regulate the activity of enzymes, receptors, and transcription factors via thiol redox control (Holmgren, 1985, 1989).

Selenite and GS-Se-SG are substrates for both reduced thioredoxin and human or calf-thymus thioredoxin reductase and NADPH. Incubation of these selenium compounds with the mammalian thioredoxin reductases in the presence of O_2 results in a large nonstoichiometric oxidation of NADPH. Selenium in the oxidation state −II is formed in these reactions (Kumar et al., 1992; Björnstedt et al., 1992, 1995). Anaerobically the oxidation of NADPH stops after the formation of selenide from selenite and GS-Se-SG.

The purpose of this study was to investigate if selenium compounds directly inhibit 5- and 15-lipoxygenase.

MATERIALS AND METHODS

Selenite and sodium borohydride were from Sigma, and Na_2S was from Merck. GS-Se-SG was prepared as described by Björnstedt et al. (1992, 1995). 15-HPETE was from Biomol (Plymouth Meeting, PA). Elemental selenium was kindly provided by Dr. Göran Zdansky, Uppsala University, Uppsala, Sweden. The human lymphoblastoid-B cell line BL41-E95-A was kindly provided by Professor George Klein, Department of Tumor Biology, Karolinska Institutet. Cells were cultivated and harvested as described by Jakobsson et al. (1992).

Preparation of Selenide. Selenide (Se^{2-}/HSe^-/H_2Se) was prepared from elemental selenium according to the method described by Klayman and Griffin (1973). *Note that selenium compounds are toxic (Olson, 1986). Avoid any contact with them! H_2Se is a toxic gas. Precaution should be taken not to inhale it.* Reaction with borohydride is exothermic with production of H_2. In studies of lipoxygenase activity selenide was purified from borate by acidification of the Se^{2-} solution and subsequently by trapping of H_2Se, in 1 M NaOH on ice as described by Verez et al. (1992). Saturated solutions (0.38 M) (Klayman & Griffin, 1973) were prepared and frozen. Selenide used in ESR studies was prepared either as described above or was made fresh 30 min before the experiment by adding $NaBH_4$ in 4:1 molar excess to 100−30 mM solutions of selenite dissolved in argon-saturated water. Under a flow of argon the reaction was complete within 20 min at room temperature.

[1] Abbreviations: TR, thioredoxin reductase; Trx, thioredoxin; Trx-S_2, oxidized thioredoxin; Trx-$(SH)_2$, reduced thioredoxin; GS-Se-SG, selenodiglutathione; Se^{2-}/HSe^-, selenide; ESR, electron spin resonance; 15-HPETE, hydroperoxyeicosatetraeinoic acid.

Enzyme Preparations. Lipoxygenase (EC 1.13.11.12), lipoxidase Type V from soybeans was purchased from Sigma Chemical Co. (St. Louis, MO) as a suspension in 2.3 M $(NH_4)_2SO_4$. Specific activities of the preparations were in accordance with the Sigma Quality Control Test Procedure *Enzymatic Assay of Lipoxidase* (Sigma Chemical Co., 1994) and was found to be higher than 0.7×10^6 units/mg (Sigma units). SDS−PAGE revealed the presence of two bands in the preparation. The main band of 70%−75% corresponds to lipoxygenase-1 (Christopher et al., 1970). In the initial experiments lipoxygenase-1 was obtained by additional purification of this commercial preparation by ion exchange chromatography on DEAE Sepharose CL 6B in a linear gradient of a phosphate buffer at pH 7.0. Pure enzyme was in the fraction eluted at phosphate concentrations above 0.16 M. Results obtained with lipoxygenase-1 were essentially the same as the ones obtained with the Sigma's crude lipoxidase type V. The reported experiments were carried out with the type V enzyme preparation without extra purification.

The suspension was centrifuged in Eppendorf tubes at 12 000 rpm for 5 min. The supernatant was discarded, and the pellet was dissolved in 50 mM Tris-HCl buffer, pH 7.5. Ammonium sulfate was removed by passage of the protein solution through Sephadex G-25, equilibrated with 50 mM Tris-HCl buffer, pH 7.5, or with 0.1 M Hepes buffer, pH 7.0, in centrifuge columns (Penefsky, 1979). The protein concentration was determined at 280 nm. The extinction coefficient of $A_{280}^{0.1\%} = 1.6$ (Petersson et al., 1987) and a molecular weight of 98 600 were used in calculations. Thioredoxin reductase from calf thymus or human placenta was purified as described by Holmgren and Björnstedt (1995).

Incubation of Sonicated Human Monoclonal B-Lymphocytes and Analysis of Leukotrienes and Monohydroxy Acids. Cells (10^7) suspended in calcium-free PBS (1 mL) containing 1 mM EDTA were sonicated twice for 5 s on ice. Preincubation with or without additives was carried out for 1 min. Thereafter, ATP was added (1 mM) and samples were kept for 30 s at 37 °C. Incubations were started by addition of $CaCl_2$ (2 mM) and arachidonic acid (40 μM). The enzymatic reaction proceeded for 10 min at 37 °C before termination by addition of 1 volume of methanol. Analysis of leukotrienes and monohydroxy acids was performed as described by Jakobsson et al. (1992).

Assay of 15-Lipoxygenase Activity after Incubation with Thioredoxin Reductase and NADPH with or without Selenite. To each of four test tubes was added 40 μL of 15-lipoxygenase (620 nM) from soybeans, which was pretreated with an equimolar amount of 15-HPETE. To the buffer containing lipoxygenase were added TR and NADPH to final concentrations of 0.5 μM and 2.8 mM, respectively, or sodium selenite to a final concentration of 1 mM, or a combination of TR, NADPH, and selenite. Then Tris-HCl, 50 mM, pH 7.5 was added to a final volume of 80 μL in all samples. The samples were preincubated for 15 min at room temperature and stored on ice. To a cuvette was added 1 mL of 0.1 M borate buffer, pH 9.0, containing arachidonic acid in a final concentration of 2.5 μM. The reactions were started by addition of 2 μL of lipoxygenase to the cuvette. Lipoxygenase activity was followed at 234 nm (Tappel et al., 1953).

Assay of 15-Lipoxygenase Activity after Anaerobic Incubation with Chemically Generated Selenide. Pure argon (AGA, Sweden) was used to purge solutions from oxygen. Argon was additionally purified from oxygen and saturated by H_2O by the passage through a fresh 0.2% solution of $Na_2S_2O_4$ in 0.1 M NaOH.

Dilutions of the stock solution of selenide were made anaerobically with 50 mM Tris-HCl, pH 7.5, which had been flushed with argon, to an estimated concentration of 35 μM. One portion of this solution was exposed to air before it was used in control experiments. Anaerobic incubation mixtures were prepared by addition of 20−50 μL of 15-lipoxygenase solution (0.09 mg/mL) to 200 μL of deoxygenated 50 mM Tris-HCl, pH 7.5, under a flow of argon. The mixture was flushed with argon for 10 min before addition of 20−50 μL of the diluted selenide solution. Argon flow was continued for 5 min at room temperature, and the sealed samples were kept on ice. To the control mixtures was added deoxygenated Tris-HCl buffer or selenide solution which had been exposed to air. Experiments similar to those with selenide were also performed with sodium sulfide. In this case 5−20 μL of 0.17 mM Na_2S solution in argon-saturated 50 mM Tris-HCl buffer, pH 7.5, was added to 70 μL of the deoxygenated protein solution, which was then incubated for 5 min at room temperature and put on ice.

Activity was measured after addition of 20−50 μL of 15-lipoxygenase, pretreated with selenide or sulfide as described above, to quartz cuvettes containing 0.70 mL of 0.20 M borate buffer, pH 9.2, and 50 μL of 0.63 mM arachidonic acid (in borate buffer). Enzyme activity was followed as the increase in absorbance at 234 nm (Tappel et al., 1953).

ESR Spectroscopy of 15-Lipoxygenase. Lipoxygenase (0.05−0.23 mM in different experiments) in 50 mM Tris-HCl buffer, pH 7.5, was incubated with 1 molar equiv of 15-HPETE dissolved in pure ethanol. To the oxidized 15-lipoxygenase was added TR, NADPH, and selenite to final concentrations of 1 μM, 5.5 mM, and 2.8 mM, respectively. Tris-HCl buffer was added to a final volume of 180 μL in all samples including controls. After incubation at room temperature for 15 min, the samples were transferred to ESR tubes and frozen in liquid nitrogen. Complexes of 15-lipoxygenase with azide were made by mixing 0.15 mM of the enzyme with 5 mM of NaN_3. In anaerobic experiments stock solutions of oxidized 15-lipoxygenase were flushed with argon and transferred into a box with N_2 atmosphere with an oxygen content of less than 0.1%. The enzyme was kept in the box in an open vial on ice for 30 min before sample preparation. Stock solutions of selenite and NADPH and Tris-HCl were treated in the same way. TR was kept in an open vial on ice in the anaerobic box. All samples were prepared inside the box and were incubated for 40 min at room temperature, transferred to ESR tubes also inside the box, sealed, and frozen in liquid nitrogen. Control samples were treated in the same manner.

ESR samples of 15-lipoxygenase preincubated with chemically generated selenide were prepared anaerobically and transferred into ESR tubes under a flow of argon. Solutions of 0.05−0.1 mM of the enzyme, in 0.1 M Hepes buffer, pH 7.0, or in 50 mM Tris-HCl buffer, pH 7.5, with 0.1 mM 15-HPETE and 0.8%−1.5% C_2H_5OH were purged from oxygen by blowing argon on the surface for 1 h at 4 °C. Fresh 30 mM selenide stock solution with or without borohydride was added to lipoxygenase, and the final selenium concentration was 0.1−8 mM. Mixtures were incubated for 10 min at room temperature, transferred into ESR tubes, and frozen. Control samples contained all the components except selenium.

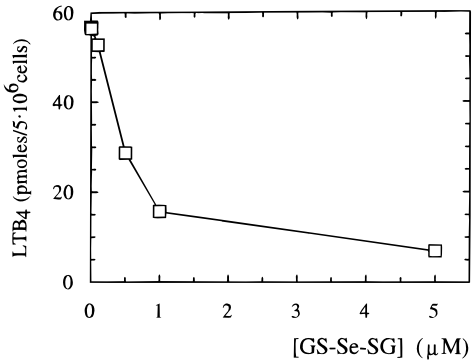

FIGURE 1: Inhibition of the synthesis of LTB_4 by GS-Se-SG. To sonicates of human B lymphocytes was added GS-Se-SG in the indicated amounts. After incubation at 37 °C for 10 min, in the presence of $CaCl_2$ (2 mM) and arachidonic acid (40 μM), the reactions were stopped and leukotriene B_4 was analyzed by HPLC.

X-band ESR spectra were recorded at 3.6−15 K on a Bruker ESP300 spectrometer equipped with a helium flow cryostat ESR-9 from Oxford Instruments. The microwave frequency was measured prior to each scan with a Hewlett Packard 5350A microwave frequency meter. Modulation frequency was 100 KHz. Intensities of components were estimated from double integrals of ESR spectra. Relative intensities of axial components, observed at $g \approx 6.1$, were measured also as ratios of magnitudes of the corresponding peaks in the absorption derivative spectra (Aasa & Vänngård, 1975). Power saturation data were collected by measuring the ESR absorption derivative peak signal intensity as a function of incident microwave power (P). In the case of $g = 4.28$ signals peak-to-peak intensities (I) of the sharpest component were measured. The half-saturation power values, $P_{1/2}$, were determined graphically plotting $(I/P^{0.5})/(I/P^{0.5})_{max}$ versus log(P). $P_{1/2}$ values are defined as abscissas of the points of crossings of linear interpolations of the dependencies at high powers with the level 0.707 (Sahlin et al., 1986).

RESULTS

Inhibition of 5-Lipoxygenase Activity in Sonicates from Human B-Lymphocytes. Addition of GS-Se-SG to sonicates of human monoclonal B-lymphocytes resulted in a decreased synthesis of LTB_4 with an apparent IC_{50} of 0.5 μM (Figure 1).

Sodium selenite also inhibited the synthesis of LTB_4 in sonicates of B lymphocytes. The levels of LTB_4 decreased from 46 pmol/5 × 10^6 cells to 7 pmol/5 × 10^6 cells after incubation with 10 μM of selenite for 10 min. Addition of thioredoxin reductase and NADPH did not inhibit the activity of 5-lipoxygenase in the sonicates (data not shown).

Inhibition of 15-Lipoxygenase Activity. To elucidate if the mechanism of action of selenium compounds was due to direct interference with lipoxygenase or indirectly mediated by cellular factors, we used isolated soybean 15-lipoxygenase since the amount of pure 5-lipoxygenase necessary for these experiments was not available.

Incubation for 15 min at room temperature of 15-lipoxygenase together with TR, NADPH, and selenite prior

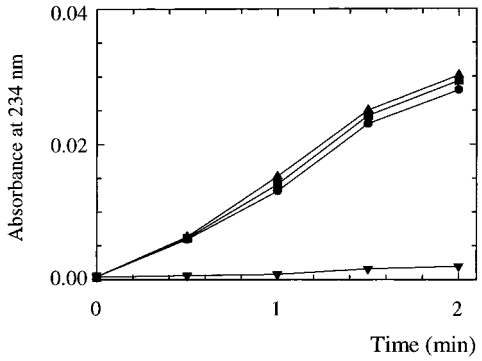

FIGURE 2: Inhibition of the activity of 15-lipoxygenase. To the cuvette containing borate buffer with 2.5 μM arachidonic acid was added lipoxygenase incubated with Tris buffer (●), TR + NADPH (▲), selenite (■), or a mixture of TR, NADPH, and selenite (▼). Final concentrations in the cuvette were as follows: 15-lipoxygenase, 0.6 nM; TR, 1 nM; NADPH, 5.6 μM; and selenite, 2 μM. The activity was followed at 234 nm.

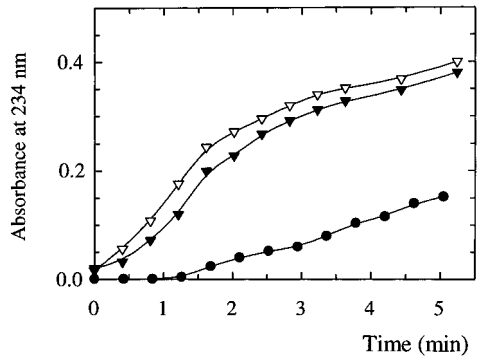

FIGURE 3: Inhibition of 15-lipoxygenase activity by selenide. To the cuvette containing 0.75 mL of 0.20 M borate buffer, pH 9.2, and 42 μM arachidonic acid was added 25 μL of 15-lipoxygenase (to a final concentration of 4 nM) preincubated for 5 min at room temperature with (A) 6 μM selenide solution anaerobically (●), (B) aerated selenide, 6 μM solution (▲), or aerated buffer (△).

to the addition of arachidonic acid resulted in inhibition of 15-lipoxygenase activity (Figure 2). The degree of inhibition was strictly dependent on the time of preincubation. No effect was observed within 1 min, and total inhibition was observed after 15 min. Addition of selenite or TR and NADPH separately did not inhibit the lipoxygenase activity (Figure 2). The results suggest that selenite must be reduced to inhibit 15-lipoxygenase activity.

Anaerobic preincubation of 15-lipoxygenase with chemically generated selenide resulted in decreased activity (Figure 3). In contrast, sodium sulfide (0.1−20 mM) did not inhibit enzyme activity (data not shown). Exposure of selenide to molecular oxygen prior to addition of the enzyme resulted in no inhibition of 15-lipoxygenase activity (Figure 3).

ESR Spectra of 15-Lipoxygenase. The ESR spectra of 15-lipoxygenase which was not pretreated with 15-HPETE did not yield any peaks (data not shown), showing that the iron in the active site was in the ferrous form (Slappendel et al., 1982). An intense peak at $g = 6.1$ and a less intense derivative-looking feature at $g = 4.28$ appeared after addition of 1 molar equiv of 15-HPETE (Figure 4A). This spectrum of deoxygenated enzyme resembles the reported spectra of oxidized 15-lipoxygenase recorded in the presence of ethanol (de Groot et al., 1975; Feiters, 1984; Petersson et al., 1987; Nelson et al., 1991; Zhang et al., 1995). The ESR spectrum changed after addition of TR, selenite, and NADPH followed

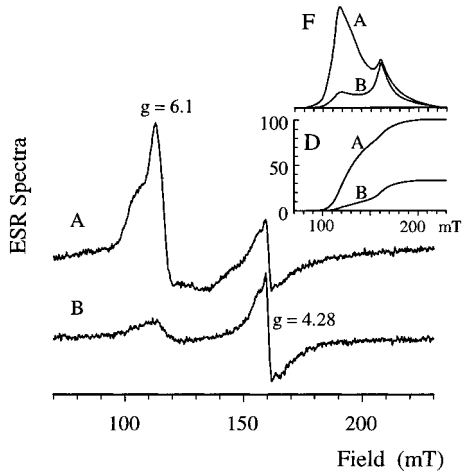

FIGURE 4: ESR spectra of oxidized lipoxygenase, ~0.1 mM, anaerobically preincubated at room temperature with 0.7 μM calf-thymus thioredoxin reductase, 4 mM Na_2SeO_3, 5 mM NADPH: (A) for less than 1 min and (B) for 15 min; argon-saturated 0.1 M Hepes buffer, pH 7.0, 0.1 mM 15-HPETE, 0.5% C_2H_5OH. Instrument parameters: microwave frequency 9615.8 MHz; microwave power, 0.49 mW; modulation amplitude, 1.1 mT; sample temperature, 3.6 K. Main panel: absorption derivative spectra; first integrals (F) of these spectra; double integrals of the spectra (D, in arbitrary units).

by anaerobic incubation at room temperature (Figure 4B). First, the intensity of the peak at $g = 6.1$ decreased, which could be due to the reduction of ferric iron or/and to a structural change of the active site. Second, the intensity of derivative looking feature at $g = 4.28$ increased, indicating a distortion of the ligation geometry of the active site. Similar changes were also observed in the presence of O_2 (data not shown). High concentrations of TR, NADPH, and selenite had to be used because of the high concentration of 15-lipoxygenase (0.12−0.23 mM in different experiments) that was needed to obtain a clear ESR signal.

The ESR experiments were performed also with lipoxygenase treated with chemically generated selenide. Incubation of 15-lipoxygenase with selenide at high concentrations, 0.4−8 mM, either anaerobically or in the presence of oxygen, with or without $NaBH_4$, resulted in disappearance of ESR signals (Figure 5A,B). The data evidence that selenide is able to reduce the ferric iron of 15-lipoxygenase. No changes in spectra occurred when samples were thawed aerobically.

Incubation of 15-lipoxygenase with a low (0.14 mM) selenide concentration reduced the intensity of the peak at $g = 6.1$, as compared to the control (Figure 6A,B). In contrast, the intensity of the peak at $g = 4.28$ increased (Figure 6A,B).

Microwave power saturation properties of the $g = 4.28$ component of the rhombic form induced by selenide poorly depended on observation temperature above 3.6 K: $P_{1/2} \approx 1.4$ mW at 3.6 K, and $P_{1/2} \approx 1.5$ mW at 6.0 K. The fact that ground state transitions from the rhombic form produced by selenide treatment were practically not visible at temperatures higher than 3.6 K and the shape of the observable signal (Gaffney & Silverstone, 1993) and its saturation behavior evidence both high rhombicity and low zero field splitting constant for these signals.

The change of the predominantly axial iron ESR signal of 15-lipoxygenase observed in the presence of ethanol to the predominantly rhombic Fe^{3+} EPR signal observed in the enzyme treated by selenite + TR + NADPH or by selenide resembles events happening in oxidized 15-lipoxygenase

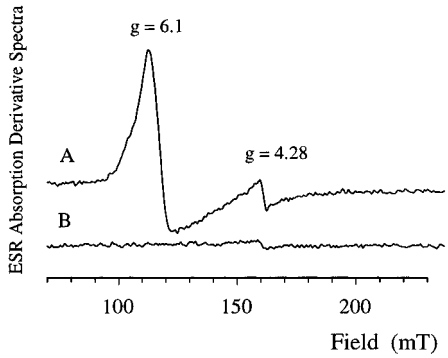

FIGURE 5: Reduction of the active site iron of lipoxygenase by selenide. The enzyme, ~0.1 mM, was oxidized by 5 min incubation with 0.4 mM of 15-HPETE and separated from 15-HPETE by gel filtration. Lipoxygenase was deoxygenated and incubated in 0.05 M Tris-HCl buffer, pH 7.5, 1.5% C_2H_5OH, at room temperature with (A) 1.6 mM of $NaBH_4$ for 5 min or (B) with 0.8 mM selenide and 1.6 mM $NaBH_4$. Instrument parameters: microwave frequency, 9616.9 MHz; microwave power, 1 mW; modulation amplitude, 1.1 mT; sample temperature, 3.8 K.

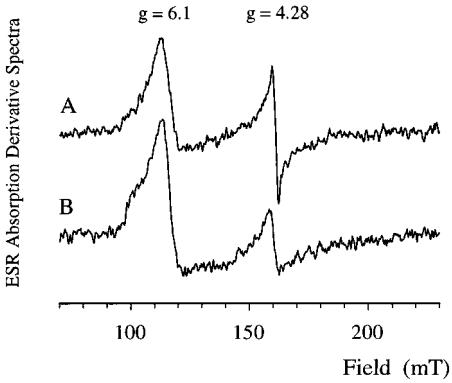

FIGURE 6: ESR spectra of oxidized 15-lipoxygenase, 0.06 mM, incubated anaerobically with (A) 0.14 mM selenide made by mixing selenite and $NaBH_4$ in 1:4 molar ratio, and (B) the control with 0.6 mM of $NaBH_4$; argon-saturated 0.1 M Hepes buffer, pH 7.0, with 0.1 mM 15-HPETE and 0.8% ethanol. Instrument parameters: microwave frequency, 9616 MHz; microwave power, 1 mW; modulation amplitude, 1.1 mT; sample temperature, 3.6 K.

upon reaction with catecholate derivatives (Galpin et al., 1976; Nelson, 1988b; Nelson et al., 1995) and with azide. We recorded ESR spectra of the azide complex of lipoxygenase (data not shown). They were similar to those reported in literature (Gaffney et al., 1993; Zhang et al., 1995). The sharp derivative-looking feature at $g \approx 4.3$ of N_3^- induced spectra was considerably broader (5.6 mT peak-to-peak width) than the corresponding rhombic form produced by selenide (3.4 mT). This azide-induced signal also was saturated at higher values of microwave power: $P_{1/2} \approx 7$ mW at 6.0 K.

DISCUSSION

This study demonstrates that a product of selenium metabolism inhibits the activity of lipoxygenase. The suggested common metabolite of inorganic selenium compounds, GS-Se-SG was a very efficient inhibitor of the synthesis of leukotrienes in sonicates of human B-lymphocytes. Selenodiglutathione is immediately reduced in the presence of thiols such as TR, NADPH, and Trx-$(SH)_2$ (Kumar et al., 1992; Björnstedt et al., 1992, 1995) or by GSH, NADPH, and glutathione reductase (Ganther, 1971), present in the sonicates from human B-lymphocytes. One product of this reduction is selenide (HSe^-/Se^{2-}).

Selenide is rapidly oxidized by molecular oxygen in air, and elemental selenium is formed (Spallholz, 1994). Preincubation of 15-lipoxygenase with chemically generated selenide under anaerobic conditions resulted in inhibition. After preincubation with aerated selenide solution, in which elemental selenium will be present, the enzyme remained fully active. These results clearly show that the compound inhibiting lipoxygenase activity is selenide. The inhibition is not reversible despite the presence of oxygen during the lipoxygenase assay with arachidonic acid. Sulfide, the analogue of selenide, caused no inhibition.

Preincubation of 15-lipoxygenase with TR, NADPH, and selenite also resulted in inhibition of the activity, while addition of TR, NADPH, and selenite to the enzyme without preincubation did not lead to inhibition. The most likely explanation for this is that selenite must be reduced to selenide to inhibit lipoxygenase. Preincubation of lipoxygenase with selenite or TR plus NADPH separately did not result in inhibition. The results indicate that the common product of the reduction of GS-Se-SG and selenite by TR was the inhibitor. The disappearance of selenide under aerobic conditions could be compensated by the permanent regeneration of selenide in the TR-catalyzed reduction (Kumar et al., 1992; Björnstedt et al., 1995).

ESR data suggest that the product of the reaction of SeO_3^{2-} and NADPH in the presence of TR is partially reducing the ferric ion of the active site of 15-lipoxygenase and at the same time changes the environment of the non-reduced Fe^{3+} so that it acquires a rhombic ESR spectrum. The fact that similar changes in ESR spectra could be produced upon incubation of lipoxygenase with chemically generated selenide strongly evidences that it directly or indirectly changes the moiety of the iron site of lipoxygenase.

ESR observations of the changes of the iron active site by selenide are complicated by the fact that Fe^{3+} ion can accept electrons from selenide and by this can be converted into ESR invisible Fe^{2+} state. Therefore, contrary to the case of a number of well-known ligands, such as N_3^-, no ESR information was obtained upon an increase of selenide concentrations. The ability of selenide to reach and reduce the active site iron of lipoxygenase may itself, at least partially, be responsible for the inhibition. Many of the potent inhibitors of lipoxygenases are known to be reductants of the active site iron (Nelson et al., 1991; Van der Zee et al., 1989).

The three-dimensional structure of soybean 15-lipoxygenase has been solved to 2.6 Å resolution (Boyington et al., 1993; Minor et al., 1993). The enzyme molecule consists of two domains. The larger domain II is carrying in its center the iron site. The iron is coordinated to four ligands in a geometry of a highly distorted octahedron (Boyington et al., 1993). Three ligands are N_ϵ of histidine residues, and the fourth is an oxygen of the COOH-terminus of the last residue in the sequence Ile-839. Two adjacent coordinate positions are unoccupied. Both of them are connected with the surface of the molecule via large internal cavities, paved predominantly by hydrophobic amino acid residues. The first channel is 18 Å long, has a conic shape with cross-section diameters of 2.5, 8, and 11 Å close to the iron, in the middle, and on the protein surface, respectively. Boyington et al. (1993) have proposed that this channel serves as a path for the

movement of molecular oxygen from the surface to the active site. The second cavity is 40 Å long. It faces the C-terminal carboxylate group and two histidine ligands and is proposed to accommodate arachidonic acid. Nelson (1988a) and Van der Heljd et al. (1992) discuss a possibility of water molecules to be iron ligands. It can easily be conceived that the active site can accommodate other exogenous ligands such as HSe^-, H_2Se, or Se° produced by TR from selenite. The two intramolecular channels proide paths for exogenous ligands. The reactive compound, selenide is widely used in chemical synthesis for the introduction of selenium into organic compounds (Behrendt & Gerwarth, 1981). The alternative to the direct iron ligation can be a modification by selenide of amino acid residues in the vicinity of the active site.

The present report describes a new mechanism by which selenium could inhibit the formation of leukotrienes. The direct action of reduced selenium compounds like selenide on lipoxygenase, in addition to the suggested indirect action via induction of selenoenzymes (Weitzel & Wendel, 1993), could at least partly explain the anti-inflammatory effects of selenium compounds.

ACKNOWLEDGMENT

We are grateful to Professor Astrid Gräslund for the opportunity to record ESR spectra and to Professor Anders Ehrenberg for encouragement and help interpreting the ESR data. We are grateful to Dr. Mats Hamberg for good advice and for his critical reading of the manuscript.

REFERENCES

Aasa, R., & Vänngård, T. (1975) *J. Magn. Reson. 19*, 308−315.

Behrendt, W., & Gerwarth, U. W. (1981) in *Gmelin Handbook of Inorganic Chemistry, 8th ed., Selenium Supplement, Vol. B1* (Tschirschnitz-Geibler, B., Ed.) pp 2−72, Springer, Berlin.

Björnstedt, M., Kumar, S., & Holmgren, A. (1992) *J. Biol. Chem. 267*, 8030−8034.

Björnstedt, M., Kumar, S., & Holmgren, A. (1995) *Methods Enzymol. 252*, 209−219.

Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B., & Zinoni, F. (1991) *Mol. Microbiol. 5*, 515−520.

Boyington, J. C., Gaffney, B., & Amzel, L. M. (1993) *Science 260*, 1482−1486.

Chasteen, N. D., Grady, J. K., Skorey, K. I., Neden, K. J., Riendeau, D., & Percival, M. D. (1993) *Biochemistry 32*, 9763−9771.

Christopher, J., Pistorius, E., & Axelrod, B. (1970) *Biochem. Biophys. Acta 198*, 12−19.

de Groot, J. J. M. C., Veldink, G. A., Vliegenthart, J. F. G., Bolding, J., Wever, R., & van Gelder, B. F. (1975) *Biochim. Biophys. Acta 377*, 71−97.

Feiters, M. C. (1984) *On the Iron in Soybean Lipoxygenases*, Ph.D. Thesis, University of Utrecht, The Netherlands.

Flohé, L. (1989) in *GSH: Chemical, Biochemical and Medical Aspects* (Dolphin, B., Poulson, R., & Avramovich, O., Eds.) Part A, pp 644−729, John Wiley & Sons, New York.

Galpin, J. R., Tielens, L. G. M., Veldink, G. A., Vliegenthart, J. F. G., & Bolding, J. (1976) *FEBS Lett. 69*, 179−182.

Gaffney, B. J., & Siverstone, H. J. (1993) in *Biological Magnetic Resonance* (Berliner, L. J. & Reuben, J., Eds.) Vol. 13, pp 1−57, Plenum Press, New York.

Gaffney, B. J., Mavrophilipos, D. V., & Doctor, K. S. (1993) *Biophys. J. 64*, 773−783.

Ganther, H. E. (1968) *Biochemistry 7*, 2898−2905.

Ganther, H. E. (1971) *Biochemistry 10*, 4089−4098.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237−271.

Holmgren, A. (1989) *J. Biol. Chem. 264*, 13963−13966.

Holmgren, A., & Björnstedt, M. (1995) *Methods Enzymol. 252*, 199−208.

Hsieh, H. S., & Ganther, H. E. (1975) *Biochemistry 14*, 1632−1636.

Jakobsson, P.-J., Odlander, B., Steinhilber, D., Rosén, A., & Claesson, H.-E. (1991) *Biochem. Biophys. Res. Commun. 178*, 302−308.

Jakobsson, P.-J., Steinhilber, D., Odlander, B., Rådmark, O., Claesson, H.-E., & Samuelsson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3521−3525.

Jakobsson, P.-J., Shaskin, P., Larsson, P., Feltenmark, S., Odlander, B., Aguilar-Santelises, M., Jondal, M., Biberfeld, P., & Claesson, H.-E. (1995) *Eur. J. Biochem. 232*, 37−46.

Kice, J. L., Lee, T. W. S., & Pan, S.-T. (1980) *J. Am. Chem. Soc. 102*, 4448−4455.

Klayman, D. L., & Griffin, T. S. (1973) *J. Am. Chem. Soc. 95*, 197−200.

Kumar, S., Björnstedt, M., & Holmgren, A. (1992) *Eur. J. Biochem. 207*, 435−439.

Kumlin, M., Dahlén, S.-E., Björck, T., Zetterström, O., Granström, E., & Dahlén, S.-E. (1992) *Am. Rev. Respir. Dis. 146*, 96−103.

Minor, W., Steczko, S., Bolin, J. T., Otwinowski, Z., & Axelrod, B. (1993) *Biochemistry 32*, 6320−6323.

Nelson, M. (1988a) *J. Am. Chem. Soc. 110*, 2985−2986.

Nelson, M. J. (1988b) *Biochemistry 27*, 4273−4278.

Nelson, M. J., Batt, D. G., Thompson, J. S., & Wright, S. W. (1991) *J. Biol. Chem. 266*, 8225−8229.

Nelson, M. J., Brennan, B. A., Chase, D. B., Cowling, R. A., Grove, G. N., & Scarrow, R. C. (1995) *Biochemistry 34*, 15219+15229.

Olson, O. E. (1986) *J. Am. Coll. Toxicol. 5*, 45−70.

Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527−530.

Percival, M. D. (1991) *J. Biol. Chem. 266*, 10058−10061.

Petersson, L., Slappendel, S., Feiters, M. C., & Vliegenthart, J. F. G. (1987) *Biochim. Biophys. Acta 913*, 228−237.

Sahlin, M., Gräslund, A., & Ehrenberg, A. (1986) *J. Magn. Reson., 67*, 135−137.

Samuelsson, B. (1983) *Science 220*, 568−575.

Samuelsson, B., & Funck, C. D. (1989) *J. Biol. Chem. 264*, 19469−19472.

Shamberger, R. J. (1985) *Mutat. Res. 154*, 29−48.

Slappendel, S., Aasa, R., Malmström, B. G., Verhagen, J., Veldink, G., & Vliegenthart, J. F. G. (1982) *Biochim. Biophys. Acta 377*, 71−79.

Spallholz, J. E. (1994) *Free Radical Biol. Med. 17*, 45−64.

Stadman, T. C. (1974) *Science, 183*, 915−921.

Stone, J., Hinks, L. J., Beasley, R., Holgate, S. T., & Clayton, B. A. (1989) *Clin. Sci. 77*, 495−500.

Tappel, A. L., Lundberg, W. O., & Boyer, P. D. (1953) *Arch. Biochem. Biophys. 42*, 293−304.

Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133−158.

Van der Heljd, L. M., Feiters, M. C., Navaratnam, S., Nolting, H.-F., Hermes, C., Veldink, G. A., & Vliegenthart, J. F. G. (1992) *Eur. J. Biochem. 207*, 793−802.

Van der Zee, J., Eling, T. E., & Mason, R. P. (1989) *Biochemistry, 28*, 8363−8367.

Verez, Z., Tsai, L., Scholz, T. D., Politino, M., Balaban, R. S., & Stadtman, T. C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2975−2979.

Weitzel, F., & Wendel, A. (1993) *J. Biol. Chem. 268*, 6288−6292.

Yamamoto, S. (1992) *Biochim. Biophys. Acta 1128*, 117−131.

Ylä-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Glass, C. K., Sigal, E., Witztum, J. L., & Steinberg, O. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6959−6963.

Zhang, Y., Gan, Q.-F., Pavel, E. G., Sigal, E., & Solomon, E. I. (1995) *J. Am. Chem. Soc. 117*, 7422−7427.

Zhang, Y.-Y., Lind, B., Rådmark, O., & Samuelsson, B. (1993) *J. Biol. Chem. 268*, 2535−2541.

Zhang, Y.-Y., Hamberg, M., Rådmark, O., & Samuelsson, B. (1994) *Anal. Biochem. 220*, 28−35.

BI9528762