Characterization of the Arachidonate and ATP Binding Sites of Human 5-Lipoxygenase Using Photoaffinity Labeling and Enzyme Immobilization

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ABSTRACT: The arachidonic acid and the ATP binding sites of human 5-lipoxygenase were characterized using photoaffinity labeling and immobilization of the enzyme on ATP-agarose. Photoaffinity labeling of the active site of 5-lipoxygenase was achieved with a novel thiopyranoindole inhibitor containing a 4-azido-3-iodobenzenesulfonyl moiety (L-708,714). This probe was found to inhibit the activity of 5-lipoxygenase ($IC_{50} = 0.3\ \mu M$) and to covalently label the enzyme after UV light irradiation. The labeling was inhibited by arachidonic acid, *N*-hydroxyurea, and dihydrobenzofuranol inhibitors which have been shown to reduce the non-heme iron center of 5-lipoxygenase. Photoaffinity labeling of 5-lipoxygenase by L-708,714 was dependent on the presence of both Ca^{2+} ions and phospholipids and was independent of ATP. It occurred at similar levels using native (Fe^{2+}), oxidized (Fe^{3+}), or H_2O_2-inactivated enzyme, but was abolished by heat inactivation of the enzyme. Competition of the labeling by various thiopyranoindoles and other inhibitors such as L-697,198, ZD-2138, and zileuton was found to be related to their inhibitory potency. Immobilized 5-lipoxygenase on ATP-agarose was found to be selectively eluted by adenine nucleotides (ATP > ADP > AMP) but not by solutions containing high salt concentrations, mild detergents, arachidonic acid, or inhibitors. 5-Lipoxygenase inhibitors were selectively retained on the immobilized enzyme and eluted by buffer containing arachidonic acid. These results provide evidence that the arachidonic acid binding site of 5-lipoxygenase is a site of interaction for the major structural classes of inhibitors of the enzyme and that the accessibility at this site is not restricted by the large steric hindrance around the ATP binding site of the immobilized enzyme.

5-Lipoxygenase is the first enzyme involved in the synthesis of leukotrienes from arachidonic acid. The enzyme catalyzes both the dioxygenation of arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE)[1] and its further conversion to leukotriene A_4 (LTA_4) (Yamamoto, 1992; Samuelsson & Funk, 1989). LTA_4 can be enzymatically hydrolyzed to produce LTB_4 or conjugated with glutathione to yield a class of potent bronchoconstrictors, the peptidoleukotrienes. Recent clinical studies have shown that the inhibition of 5-lipoxygenase activity, either by inhibitors of the 5-lipoxygenase reaction or indirectly by molecules that bind to the 5-lipoxygenase-activating protein (FLAP), results in a significant improvement of symptoms during cold air, exercise, or antigen-induced asthma (Israel et al., 1990; Friedman et al., 1993).

Human 5-lipoxygenase contains an essential non-heme iron atom (Percival, 1991) which undergoes reversible oxidation between the Fe^{3+} and Fe^{2+} states (Chasteen et al., 1993). The enzyme shows about 60% homology in primary sequence with 15-lipoxygenases (Sigal, 1991), and its oxygenase reaction appears to follow a basic mechanism similar to that proposed for the latter enzyme (Ford-Hutchinson et al., 1994). However, the 5-lipoxygenase shows a number of distinctive regulatory features such as a rapid inactivation during catalysis (Rouzer et al., 1988) and a dependence on Ca^{2+} ions and ATP for maximal activity (Aharony & Stein, 1986; DeWolf, 1991). The magnitude of the stimulatory effect of ATP has been reported to vary widely depending on the enzyme source used, from 2−5-fold for the porcine (Ueda et al., 1986; Riendeau et al., 1989), guinea pig (Furukawa et al., 1984), and human (Percival et al., 1992) enzymes to 300-fold for the rat 5-lipoxygenase (Hogaboom et al., 1986). The mechanism by which ATP increases enzymatic activity is not understood and does not require hydrolysis of the phosphodiester bond since ADP and AMP can also stimulate the reaction. The primary amino acid sequence of the 5-lipoxygenase did not provide any evidence for a nucleotide binding site on the enzyme (Dixon et al., 1988; Balcarek et al., 1988, Matsumoto et al., 1988).

Both the cellular synthesis of leukotrienes and the activity of 5-lipoxygenase are stimulated by various agents that cause an increase in intracellular calcium concentration. Ca^{2+} ions promote the association of the enzyme to cellular membranes (Rouzer & Kargman, 1988) or to phospholipid vesicles in vitro (Wong & Crooke, 1991). In addition, the enzyme activity has been shown to be a function of the arachidonate to phospholipid ratio instead of the bulk substrate concentration, suggesting that the 5-lipoxygenase reaction is occurring at the interface of the phospholipid vesicles (Riendeau et

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[1] Abbreviations: CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CPHU, *N*-(4-chlorophenyl)-*N*-hydroxy-*N*′-(3-chlorophenyl)urea; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FLAP, 5-lipoxygenase activating protein; 5-HPETE, 5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid; HPLC, high-pressure liquid chromatography; 13-HPOD, 13-hydroperoxyoctadeca-9,11-dienoic acid; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; THF, tetrahydrofuran.

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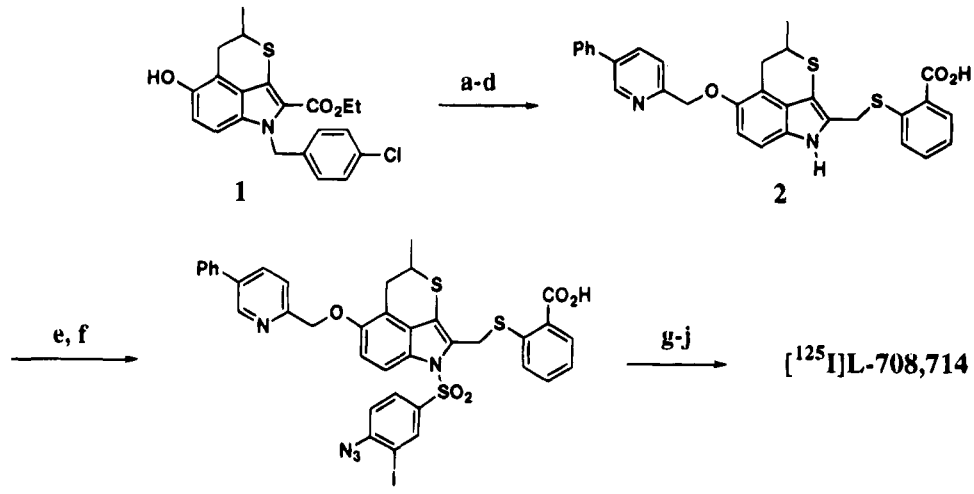
Scheme 1:[a] Synthesis of L-708,714



L-708,714

[a] Reagents: (a) BBr_3, CH_2Cl_2; (b) Cs_2CO_3, MeCN, 5-phenyl-2-picolylchloride; (c) $LiAlH_4$, THF; (d) thiosalicylic acid, $BF_3{\cdot}Et_2O$, dichloromethane; (e) potassium bis(trimethylisilyl)amide, THF; (f) 4-azido-3-iodophenylsulfonyl chloride; (g) diazomethane; (h) hexamethylditin, tetrakis(triphenylphosphine)palladium, 1,4-dioxane, 80 °C; (i) [^{125}I]NaI, chloramine-T, DMF; (j) NaOH(aq), THF, MeOH, 65 °C.

al., 1993; Noguchi et al., 1994). Because of the short half-life of 5-lipoxygenase under the assay conditions ($t_{1/2}$ = 60–80 s) and the complexity of the catalysis in vesicle systems, steady-state kinetics with 5-lipoxygenase have proven to be extremely difficult. Most of the previous studies on 5-lipoxygenase inhibition have focused on the evaluation of the ability of the inhibitors to function as reducing agents and iron ligands (Summers et al., 1987; Riendeau et al., 1991; Falgueyret et al., 1992, McMillan & Walker, 1992). Recently, selective and orally active inhibitors of leukotriene biosynthesis displaying chiral specificity for the inhibition of 5-lipoxygenase activity have been described (McMillan et al., 1990; Bird et al., 1992; Hutchinson et al., 1994). One of these series, the thiopyranoindole derivatives, has been shown to inhibit the enzyme through a nonredox mechanism, most likely by the formation of a reversible dead-end complex with the enzyme (Falgueyret et al., 1993). In the present study, we have used a radiolabeled photoaffinity probe derived from this class of inhibitor as an alternative approach to enzyme kinetic studies for the study of the interaction between various ligands and the active site of 5-lipoxygenase. The results on the competition of the photoaffinity labeling suggest that inhibitors from different structural classes, including ZD-2138, which is under clinical evaluation (McMillan et al., 1992), and zileuton, which has shown clinical efficacy in asthma (Israel et al., 1990), interact with the arachidonic acid binding site of 5-lipoxygenase. In addition, we have shown that the noncovalently immobilized enzyme on ATP-agarose retains the ability to bind active site ligands which can subsequently be displaced by arachidonic acid.

MATERIALS AND METHODS

Chemicals and Reagents. Adenosine 5′-triphosphate-agarose (attached to beaded agarose through C_8 with a six-carbon spacer (1.1–2.4 μmol of ATP/mL of gel) and L-α-phosphatidylcholine (type III from egg yolk) were purchased from Sigma. Thiopyranoindole inhibitor L-689,065 (Hutchinson et al., 1993), L-702,701 and L-705,302 (Girard et al., 1994), L-702,590 and L-697,198 (Ducharme et al., 1994), CPHU (Falgueyret et al., 1992), L-670,630 (Lau et al. 1992), ZD-2138 (Crawley et al., 1992), and zileuton (Carter et al. 1991) were synthesized at the Dept. of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research.

Synthesis of L-708,714. Step 1: Desbenzylindole. To a solution of the previously described (Hutchinson et al., 1994) ethyl ester **1** (5.63 g, 14.6 mmol) in dichloromethane (130 mL) at 0 °C under nitrogen was added BBr_3 (1.0 M in dichloromethane, 21 mL). After being stirred for 1.5 h at 0 °C, the reaction was poured into saturated sodium bicarbonate and crushed ice. Extractive workup with dichloromethane followed by purification using flash chromatography on silica (ethyl acetate/hexane, 40:60), and crystallization from ether/hexane gave the desbenzylindole (2.9 g, 84%).

Step 2: Phenylpyridylindole. A solution of the phenolic indole from step 1 (2.5 g, 9.6 mmol), 5-phenyl-2-picolyl chloride (1.9 g, 9.6 mmol), and cesium carbonate (3.7 g, 11.6 mmol) in acetonitrile (50 mL) was stirred overnight at 25 °C under nitrogen. The mixture was poured into water, and an extractive workup with ethyl acetate and THF was followed by trituration with ether/ethyl acetate to afford a white solid (2.7 g, 64%).

Step 3: Indolyl Alcohol. The ethyl ester from step 2 (1.0 g, 2.3 mmol) was added directly to lithium aluminum hydride (180 mg, 4.7 mmol) in THF (20 mL) at 0 °C, under a nitrogen atmosphere. After being stirred for 30 min, the reaction was quenched with 2 N NaOH(aq) followed by extractive workup with dichloromethane. After trituration with ether, the resulting alcohol (0.74 g, 85%) was isolated by filtration.

Step 4: Benzoic Acid Derivative **2**. A solution of the alcohol from step 3 (400 mg, 1.0 mmol) and thiosalicylic acid (191 mg, 1.2 mmol) in dichloromethane (20 mL) was treated with boron trifluoride etherate, (153 μL, 1.2 mmol) at 25 °C under nitrogen. Stirring was continued for 10 min, and the reaction was quenched with water (10 mL); THF was then added to dissolve the solid. The organic layer was dried over magnesium sulfate and concentrated. Flash chromatography [silica, dichloromethane/methanol/ammo-

nium hydroxide(conc) 40:10:1] gave **2** as a white solid (139 mg, 27%).

Step 5: L-708,714. Potassium bis(trimethylsilyl)amide (0.80 mL, 0.5 M in toluene, 0.4 mmol) was added to a suspension of **2** (100 mg, 0.19 mmol) in THF (7 mL) at −78 °C under a nitrogen atmosphere. The mixture was stirred at −78 °C for 5 min and then at 0 °C for 1 h. Cooling the resulting solution to −78 °C was followed by addition of 4-azido-3-iodobenzenesulfonyl chloride (69 mg, 0.20 mmol) (Mais et al., 1991) in THF (1 mL). After being stirred at −78 °C for a further 30 min, the reaction was quenched with saturated ammonium chloride solution and partitioned between ethyl acetate and dilute HCl(aq). The organic layer was washed with water and brine, dried over magnesium sulfate, and concentrated. Purification on flash chromatography (silica, dichloromethane/methanol/ammonium hydroxide(conc) 60:10:1) followed by trituration with ether afforded L-708,714 as a white solid (107 mg, 57%): mp 170−180 °C (dec); ^{1}H NMR (250 MHz, DMSO-d_6) δ 1.32 (d, 3H, J = 7.3 Hz), 2.70 (dd, 1H, J = 16.9, 9.0 Hz), 3.24−3.40 (m, 2H), 4.46 (d, 1H, J = 13.0 Hz), 4.54 (d, 1H, J = 13.0 Hz), 5.24 (d, 1H, J = 13.5 Hz), 5.30 (d, 1H, J = 13.5 Hz), 7.21 (d, 1H, J = 9.0 Hz), 7.22 (m, 1H), 7.37 (d, 1H, J = 8.5 Hz), 7.37−7.61 (m, 5H), 7.61 (d, 1H, J = 7.9 Hz), 7.70−7.79 (m, 2H), 7.79 (d, 1H, J = 8.5 Hz), 7.56 (d, 1H, J = 7.3 Hz), 7.93 (dd, 1H, J = 8.5, 2.8 Hz), 8.12 (dd, 1H, J = 9.0, 2.8 Hz), 8.17 (d, 1H, J = 2.8 Hz), 8.89 (d, 1H, J = 2.8 Hz).

Synthesis of [^{125}I]L-708,714. Conversion of the ^{127}I in L-708,714 to ^{125}I was accomplished following the methodology of Mais et al. (1991). Prior to preparing the radiolabeled material, a larger scale conversion of the aryltrimethyltin compound to L-708,714 was performed using sodium [^{127}I]-iodide and the identity of the product was confirmed by NMR and TLC.

Step 1: Methyl Ester. A solution of L-708,714 (24 mg) in THF (1 mL) at 25 °C was treated with excess diazomethane in ether. The excess diazomethane was quenched with acetic acid and the mixture was diluted with ethyl acetate, washed with sodium bicarbonate, dried (magnesium sulfate) and concentrated. Purification on silica (ethyl acetate/hexane 40:60) followed by trituration with ether gave the methyl ester of L-708,714 (19 mg, 78%).

Step 2: Aryltrimethyltin. The methyl ester (10 mg, 12 μmol) from step 1 and tetrakis(triphenylphosphene)palladium (0.5 mg, 0.4 μmol) in 1,4-dioxane (2 mL) under nitrogen were frozen at 0 °C, evacuated and flushed with nitrogen three times, and then thawed at 25 °C. The freeze−thaw process was repeated three times. Hexamethylditin (5 μL, 23 μmol) was then added, and the mixture was heated at 80 °C for 1 h. After cooling, the solvent was evaporated and the residue was purified on silica (ethyl acetate/hexane 30:70) followed by crystallization from ether to yield the aryltrimethyltin compound as a white solid (5 mg, 48%): ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.22 (s, 9H), 1.38 (d, 3H, J = 7.3 Hz), 2.76 (dd, 1H, J = 16.9, 10.2 Hz), 3.26 (m, 1H), 3.39 (dd, 1H, J = 16.9, 2.8 Hz), 3.88 (s, 3H), 4.52 (d, 1H, J = 13.0 Hz), 4.65 (d, 1H, J = 13.0 Hz), 5.28 (s, 2H), 7.00 (d, 1H, J = 8.5 Hz), 7.06 (d, 1H, J = 8.5 Hz), 7.22 (m, 1H), 7.40−7.63 (m, 8H), 7.26 (d, 1H, J = 2.8 Hz), 7.82−7.96 (m, 4H), 8.86 (d, 1H, J = 2.8 Hz).

Step 3: [^{125}I]L-708,714 Methyl Ester. A solution of the aryltrimethyltin compound (0.1 mg, 0.1 μmol) was dissolved in DMF (200 μL) at 25 °C. It was necessary to run the iodination reaction in DMF instead of methanol, as reported by Mais et al. (1991), due to the low solubility of the aryltrimethyltin in methanol. Sodium [^{125}I]iodide (2 mCi/40 μL, aqueous) was added followed by chloramine-T (10 μL, 0.02 M in DMF). The mixture was stirred for 2 h followed by addition of 2-mercaptoethanol (0.5 μL). After being stirred for an additional 30 min, the entire reaction mixture was injected directly onto a Nova-pak C_{18} column (1 cm diameter by 10 cm) and eluted with a mixture of acetonitrile/water (80:20) containing 0.1% acetic acid and 0.002% mercaptoethanol. With a solvent flow rate of 2 mL/min the ^{125}I methyl ester derivative eluted at 19−20 min. The corresponding ^{127}I methyl ester (unlabeled), from step 1, coeluted with the obtained radiolabeled compound. The obtained radiochemical yield based on ^{125}I was 41%.

Step 4: [^{125}I]L-708,714. The HPLC fractions containing the product from step 3 were evaporated to dryness on a Rotovap, and the residue was dissolved in THF (120 μL) and methanol (120 μL). After sodium hydroxide was added (60 μL, 1 M aqueous), the solution was flushed with nitrogen and heated to 65 °C for 35 min under a nitrogen atmosphere. The solution was cooled to 25 °C, glacial acetic acid (50 μL) was added and the total volume was reduced to about 130 μL by gently blowing nitrogen over the solution while stirring. Dilution of the mixture with eluent (100 μL) was followed by injection on HPLC, using the same conditions as in step 3. The [^{125}I]L-708,714 eluted at 7.8 min and also coeluted with [^{127}I]L-708,714 (unlabeled) when they were co-injected. After purification, the radiochemical yield of the hydrolysis reaction was 50%. Evaporation of the eluent solvent and redissolving the radiolabeled compound in 1 mL of ethanol containing 0.002% mercaptoethanol provided a solution that was stored at −80 °C for up to 3 months without significant chemical degradation.

Purification of Recombinant Human 5-Lipoxygenase. Human 5-lipoxygenase was obtained from a baculovirus insect cell (Sf9) expression system and affinity-purified on ATP-agarose using a modification of the previously described procedure (Denis et al.,1991; Percival et al., 1992). Typically, the 100 000g supernatant (S100) from a cellular homogenate (500 mL, 2−7 mg of protein/mL, 1.5−4 μmol of 5-HPETE/mg of protein) was loaded on a 10 mL column of ATP-agarose (1.1 μmol of ATP/mL of gel) equilibrated with 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA at 4 °C (buffer A). The column was then washed with 100 mL of buffer A followed by 0.5 M NaCl in buffer A (150 mL). After reequilibration of the column in buffer A (75 mL), 5-lipoxygenase was eluted with 20 mM ATP in buffer A. The enzymatic activity of each fraction was determined spectrophotometrically and the protein content was measured using a dye binding assay (Bio-Rad). The purified enzyme had a specific activity of 10−76 μmol of 5-HPETE/mg of protein and was kept in liquid nitrogen for a period up to 1 year without measurable loss of activity.

Measurement of 5-Lipoxygenase Activity. For the assessment of inhibitor potency, the enzymatic activity of 5-lipoxygenase was measured spectrophotometrically using the S100 fraction from Sf9 cells infected with the recombinant baculovirus and an incubation mixture containing 50 mM sodium phosphate, pH 7.4, 12 μg/mL phosphatidylcholine, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 20 μM arachidonic acid. Inhibitors were added from a concentrated solution in DMSO (0.2% final DMSO concentration). The reaction was initiated

by the addition of an aliquot of the enzyme preparation, and the rate of conjugated diene formation was followed at room temperature for 2 min. Enzymatic activity was calculated from the highest linear rate of the diene formation (A_{234}), and percentage of inhibition was calculated relative to a control reaction containing the DMSO vehicle. Measurement of the activity of the purified enzyme was performed using an incubation mixture containing 50 mM sodium phosphate, pH 7.4, 12 μg/mL phosphatidylcholine, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.3 mM $CaCl_2$, 20 μM arachidonic acid, and 0.1 or 0.2 mM ATP (Percival et al., 1992). The half-time of product formation in this assay is about 60−80 s. The activity of 5-lipoxygenase is expressed as the amount of 5-HPETE accumulated after an incubation of 400 s at room temperature using an ϵ of 23 000 M^{-1} cm^{-1} at 238 nm (Gibian & Vanderberg, 1987).

Photoaffinity Labeling. The photoaffinity labeling was performed by incubation of either the S100 fraction (10−15 μg of protein) or purified human 5-lipoxygenase (0.1−0.5 μg of protein) with [^{125}I]L-708,714 (0.4−0.5 μCi) at a final concentration of 10 nM. The reaction was carried out in 100 μL of buffer containing 50 mM sodium phosphate, pH 7.4, 12 μg/mL phosphatidylcholine, 0.2 mM ATP, 0.2 mM $CaCl_2$ and 0.1 mM EDTA. Inhibitors were added from a concentrated solution in DMSO (1% final DMSO concentration). The reaction mixture was then exposed under UV light (UVGL-25 Mineralight at 254 nm suspended 3 cm above the samples) for 3 min at room temperature. After the UV light exposure, 50 μL of electrophoresis sample buffer (0.1 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 10% β-mercaptoethanol, 30% glycerol, 0.05% bromophenol blue) was added. Proteins were resolved by 10% polyacrylamide gel electrophoresis (Laemmli, 1970). Gels were dried and exposed to Kodak XAR-2 film at −70 °C for 24−48 h. The incorporation of ^{125}I into 5-lipoxygenase was quantitated by scanning of autoradiograms using an LKB 2202 Ultroscan laser densitometer.

Batch Elution of 5-Lipoxygenase. The ability of detergents and adenine nucleotides to elute 5-lipoxygenase from ATP-agarose was assessed by a batch elution method. Briefly, 150−400 μL of a suspension of ATP-agarose gel was mixed with 150−200 μL of the S100 fraction for 30−60 min at 4 °C to allow adsorption of the enzyme. The mixture was then centrifuged (14 000*g* for 4 min), and the supernatant removed and replaced by an equivalent volume of buffer A containing detergents or nucleotides. After an incubation of 30−60 min, the gel was pelleted by centrifugation and the supernatant was tested for 5-lipoxygenase activity. The 5-lipoxygenase remaining adsorbed on the gel was determined by resuspending the gel in buffer A containing 20 mM ATP, centrifugation, and assay of enzymatic activity.

Inhibitor Binding to Immobilized 5-Lipoxygenase. Purified 5-lipoxygenase was freed of ATP by gel filtration on a PD-10 column (Pharmacia) in buffer A. The enzyme (380 μL, 5 nmol) was loaded on a column of ATP-agarose (400 μL, 0.44 μmol of ATP) equilibrated in buffer A and washed with 6 mL of buffer A containing 5 mM CHAPS and 0.15 M NaCl (buffer B) to reduce the nonspecific binding of the inhibitor. A solution of 1 mL containing L-697,198 and indomethacin at a concentration of 1 μM in buffer B was applied, and the column washed with 4 mL of buffer B, followed by 2 mL of buffer B containing 100 μM arachidonic acid. After a wash with 8 mL of buffer B, the 5-lipoxygenase was eluted with a solution of 20 mM ATP in buffer B. Fractions of 500 μL were collected and analyzed for protein content and level of inhibitor by RP-HPLC on a Nova-pak C_{18} column using a mixture of acetonitrile/water (60:40) as a solvent system for L-697,198 and a mixture of acetonitrile/water/acetic acid (50:50:0.5) for indomethacin. The amounts of L-697,198 and indomethacin were quantitated from the peak area at 254 and 240 nm, respectively, relative to that of known amounts of standards.

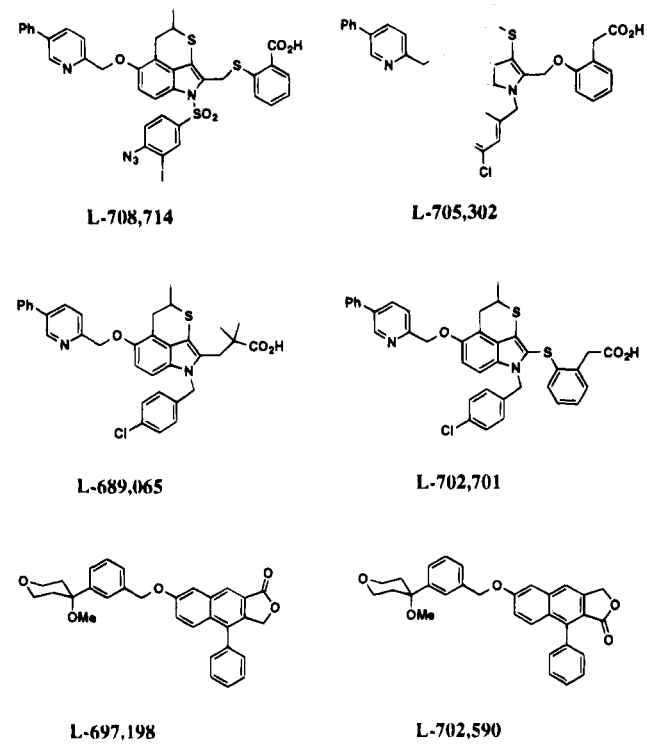


FIGURE 1: Stuctures of thiopyranoindole and naphthalenic lignan inhibitors of 5-lipoxygenase.

RESULTS

Characteristics of Photoaffinity Labeling of 5-Lipoxygenase. A thiopyranoindole derivative containing a 4-azido-3-iodobenzenesulfonyl group was synthesized as a photoaffinity probe for 5-lipoxygenase (L-708,714, structure shown in Figure 1). The 4-azido-3-iodobenzenesulfonyl group in L-708,714 was chosen because it has been successfully used in photoaffinity probes for other eicosanoid binding proteins (Mais et al., 1991). In addition, the ^{127}I on this functional group can be easily converted to ^{125}I via the trimethyltin derivative. L-708,714 functioned as a direct inhibitor of 5-lipoxygenase in the absence of photolysis with an IC_{50} value of 0.3 μM. The reversibility of this inhibition was investigated by a dilution experiment in which 20 nM 5-lipoxygenase was preincubated with 0.3 μM L-708,714 for 1 min and diluted 10-fold prior to the measurement of enzymatic activity. No inhibition of activity was observed after dilution as compared to a 63% inhibition observed in the control for the inhibition of the enzyme (2 nM) by 0.3 μM L-708,714, indicating that the inhibition by the probe is mainly due to reversible binding to the enzyme in the absence of the photoreaction. When incubated with soluble extracts of Sf9 cells containing human 5-lipoxygenase, photolysis of [^{125}I]L-708,714 resulted in the very selective labeling of a

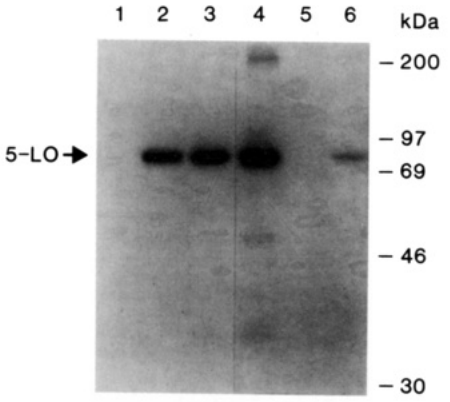


FIGURE 2: Photoaffinity labeling of 5-lipoxygenase by [^{125}I]L-708,714. Photoaffinity labeling was performed using the S100 fraction and 10 nM [^{125}I]L-708,714 in the presence of Ca^{2+} and phosphatidylcholine as described under Materials and Methods. The proteins were resolved by SDS−polyacrylamide gel electrophoresis and radiolabeled 5-lipoxygenase was detected by autoradiography. The labeling of 5-lipoxygenase was determined for samples not irradiated (lane 1) or irradiated for 10 s, 1 min, and 3 min (lanes 2−4, respectively). The probe was preirradiated for 3 min before the addition of the S100 fraction and the sample analyzed without further treatment (lane 5) or after a second irradiation of 3 min (lane 6).

protein band corresponding to 5-lipoxygenase which is detected after SDS−polyacrylamide gel electrophoresis (Figure 2). The labeling of the protein was rapid upon photolysis and increased only slightly when the reaction time was increased from 10 s to 3 min (Figure 2, lanes 2−4). Control experiments showed that no labeling of the protein was detected when the UV-irradiation step was omitted or when the radiolabeled probe was photolyzed before addition of the enzyme (Figure 2, lanes 1 and 5, respectively). A second photolysis after addition of fresh enzyme resulted only in a low level of labeling (5%) indicating that the probe was extensively degraded under the irradiation conditions used (Figure 2, lane 6).

The photoaffinity labeling of 5-lipoxygenase was inhibited by the potent inhibitor L-705,302 with an IC_{50} value of 0.1 μM (Figure 3), a value slightly higher than that obtained for inhibition of the enzymatic activity ($IC_{50} = 0.02\ \mu$M). Some level of covalent labeling ranging from 0 to 20% of the maximal incorporation for different experiments could not be eliminated by nonradiolabeled inhibitors and may represent nonspecific labeling by [^{125}I]L-708,714 or its photolysis degradation products. Arachidonic acid reduced the level of photoaffinity labeling dose-dependently with an IC_{50} value of 43 μM (Figure 3).

The enzymatic reaction catalyzed by purified 5-lipoxygenases involves a Ca^{2+}-dependent adsorption to phospholipid vesicles and exhibits the characteristics of interfacial catalysis. The effect of Ca^{2+} and phosphatidylcholine on the photoaffinity labeling of 5-lipoxygenase was determined under the conditions of the enzyme assay where these molecules cause a marked stimulation of the activity. Photoaffinity labeling of the protein in the absence of phosphatidylcholine was not affected by the presence of either EDTA or Ca^{2+} ions (Figure 4, lanes 1−3). The addition of phosphatidylcholine to the incubation mixture reduced the level of labeling, an effect that was eliminated by the presence of Ca^{2+} (Figure 4, lanes 4-6). These results can be explained by a partitioning of the probe into the phosphatidylcholine vesicles, thereby reducing its effective

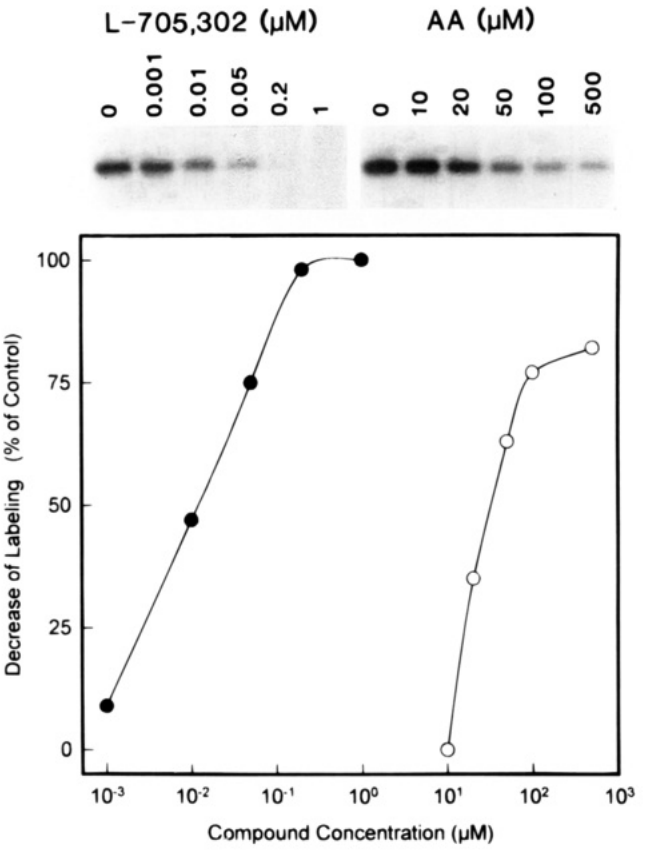


FIGURE 3: Competition of the photoaffinity labeling of 5-lipoxygenase by L-705,302 and arachidonic acid. The S100 fraction was incubated with 10 nM [^{125}I]L-708,714 and the indicated concentration of L-705,302 (●) or arachidonic acid (AA) (○) prior to photolysis and electrophoresis.

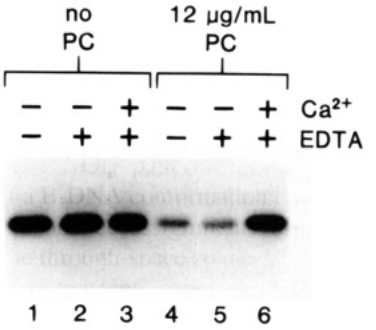


FIGURE 4: Dependence of the photoaffinity labeling of 5-lipoxygenase on Ca^{2+} ions. Purified human 5-lipoxygenase was photoaffinity labeled by [^{125}I]L-708,714 in the absence (lanes 1−3) or in the presence of 12 μg/mL phosphatidylcholine (PC) (lanes 4−6). EDTA and Ca^{2+} ions were added at a final concentration of 0.1 and 0.3 mM, respectively.

concentration and Ca^{2+} causing a stimulation in labeling by favoring the adsorption of the enzyme to the vesicles.

The effect of inactivation of 5-lipoxygenase on the level of photoaffinity labeling was also investigated. Figure 5 shows that a similar level of labeling was observed after inactivation of the purified enzyme by pretreatment with H_2O_2 (lanes 1 and 3) and that the labeling was competitively inhibited by L-705,302 in each case (lanes 2 and 4). No significant difference in labeling was observed between the Fe^{2+} enzyme form and the 13-HPOD-oxidized Fe^{3+} form (data not shown). In contrast, no significant photoaffinity labeling was detected for the heat-inactivated enzyme (Figure 5, lane 5).

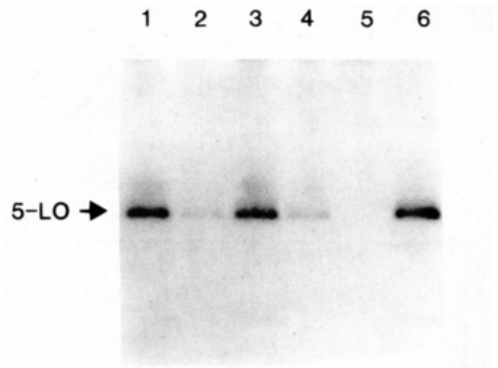


FIGURE 5: Effect of enzyme inactivation on the photoaffinity labeling of 5-lipoxygenase (5-LO). Purified 5-lipoxygenase (0.5 μg) was photoaffinity labeled using (A) active 5-lipoxygenase (11 μmol of 5-HPETE/mg of protein) in the absence (lanes 1 and 6) and in the presence of 1 μM L-705,302 (lane 2), (B) H_2O_2-inactivated enzyme in the absence (lane 3) or in the presence of 1 μM L-705,302 (lane 4), and (C) heat-inactivated enzyme (lane 5).

Table 1: Inhibition of Photoaffinity Labeling and of 5-Lipoxygenase Activity[a]

	IC_{50} (μM)	
inhibitors	photoaffinity labeling	5-lipoxygenase activity
L705,302	0.1	0.02
L-689,065	0.4	0.3
L-702,701	10	1.5
L-697,198	0.5	0.02
L-702,590	>10	>6
ZD2138	0.4	0.3
CPHU	1.2	0.1
L-670,630	1	0.4
zileuton	10	10

[a] The effect of the various inhibitors (structures in Figure 1) was determined using the S100 fraction as a source of 5-lipoxygenase. Each IC_{50} value is an average of at least two different titrations.

Competition of Photoaffinity Labeling. Selected compounds from the different classes of 5-lipoxygenase inhibitors were investigated for their ability to compete for the photoaffinity labeling of the enzyme by L-708,714. The IC_{50} values for the inhibition of the labeling are compared to those obtained for the inhibition of enzymatic activity (Table 1). The various thiopyranoindole analogs inhibited in both assays according to their rank order of potency although the compounds tend to be more potent at inhibiting the enzymatic reaction (5−7-fold). Interestingly, compounds from different structural classes such as the 2-quinolone inhibitor ZD-2138 and the *N*-hydroxyurea zileuton competed for photoaffinity labeling with IC_{50} values similar to those measured for the inhibition of enzymatic activity (IC_{50} values of 0.3 and 10 μM for ZD-2138 and zileuton, respectively). In the series of naphthalenic lignans, L-697,198 was an effective competitor of the labeling (IC_{50} = 0.5 μM) but a more potent inhibitor of enzyme activity (IC_{50} = 0.02 μM). L-702,590, an inactive analog of L-697,198 (Figure 1), did not affect the photoaffinity labeling of 5-lipoxygenase (Table 1). L-670,630 and CPHU are inhibitors of the lipoxygenase reaction, which can reduce the non-heme iron center of the enzyme (Falgueyret et al., 1992; Chasteen et al., 1993). These compounds were also able to compete for the photoaffinity labeling of 5-lipoxygenase at concentrations 2−10-times higher than those required to inhibit the enzymatic activity (Table 1), suggesting that the binding of the thiopyranoindole probe occurs in the proximity of the active site iron of the enzyme.

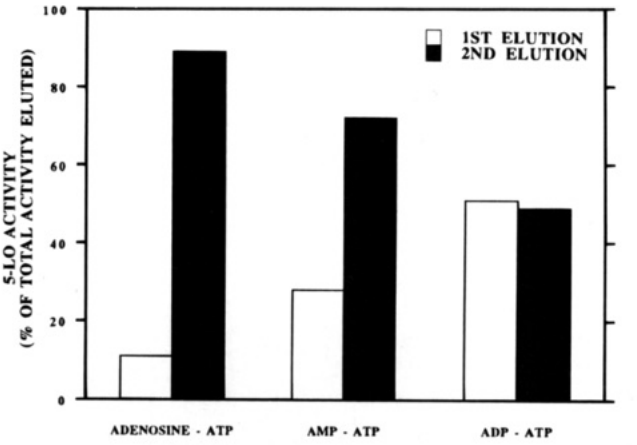


FIGURE 6: Selectivity of adenine nucleotides for the elution of immobilized 5-lipoxygenase. The ability of nucleotides to elute 5-lipoxygenase from ATP-agarose was studied by the batch elution method. After a first elution cycle in the presence of the nucleotide at a concentration of 20 mM, the supernatant was removed and tested for 5-lipoxygenase activity. Elution buffer containing 20 mM ATP was then applied to release any remaining enzyme from the gel. The results are expressed as the percent of total 5-lipoxygenase activity eluted from the gel by each elution cycle.

Table 2: Stimulation of 5-Lipoxygenase Activity by Adenine Nucleotides

nucleotide	5-lipoxygenase activity (%)	nucleotide	5-lipoxygenase activity (%)
ATP	100	ATP-γ-S	80 ± 6 (n = 4)
no ATP	58 ± 2 (n = 21)	ATP-α-S	118 ± 11 (n = 4)
ADP	74 ± 2 (n = 7)	ADP-β-S	82 ± 2 (n = 3)
AMP	68 ± 4 (n = 3)	AMP-PNP	77 ± 5 (n = 4)
adenosine	58 ± 4 (n = 2)		

[a] The effect of each nucleotide on the activity of purified 5-lipoxygenase was determined at a concentration of 0.1 mM. Results are expressed as the percentage of the product accumulation observed with ATP (mean ± SEM).

Properties of the ATP Binding Site of 5-Lipoxygenase. The noncovalently immobilized 5-lipoxygenase on ATP-agarose was used to characterize the properties of the ATP binding site and its relationship to the inhibitor/arachidonate binding site of the enzyme. Using a batch elution procedure, the 5-lipoxygenase−ATP-agarose complex was found to be stable in the presence of 0.1% CHAPS or 0.1% deoxycholic acid in the absence or the presence of 0.2 M NaCl (<10% of 5-lipoxygenase eluted as compared to buffer containing ATP). The efficiency of various nucleotides at causing elution of 5-lipoxygenase from the ATP-agarose was compared by performing a first wash with 20 mM nucleotide followed by a second wash with 20 mM ATP to release the remaining bound enzyme. Under the conditions used, ADP, AMP, and adenosine released 50, 30, and 10% of the total enzymatic activity that was eluted by the two washes (Figure 6). The same order of effectiveness of adenosine nucleotides was observed for the stimulation of 5-lipoxygenase activity (Table 2). Other nucleotides GTP, UTP, and TTP (0.1 mM) were ineffective at stimulating 5-lipoxygenase activity or eluting the enzyme from ATP-agarose (20 mM) under conditions where ATP showed a maximal effect (data not shown). The ability of stable analogs of ATP to stimulate the enzyme activity was also examined (Table 2). About 80−120% of the maximal response with ATP was observed

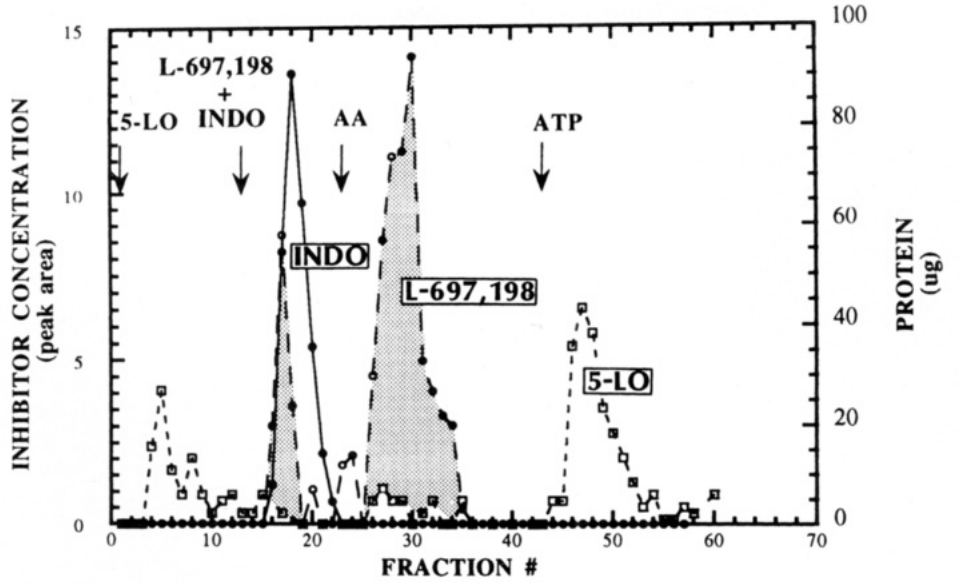


FIGURE 7: Selective binding of inhibitor by the 5-lipoxygenase immobilized through its ATP binding site. A mixture of L-697,198 and indomethacin was applied to an ATP-agarose gel column containing immobilized 5-lipoxygenase followed by subsequent washes with buffers containing 100 μM arachidonic acid (AA) and ATP. The fractions were analyzed for the content in L-697,198, indomethacin and 5-lipoxygenase protein. Total recoveries for indomethacin and L-697,198 were of 92 and 20%, respectively.

with the various analogs, further demonstrating that the hydrolysis of a phosphodiester bond is not required for the stimulation of 5-lipoxygenase activity.

The characteristics of the stimulation by ATP suggest that ATP functions as an allosteric effector and that the active site might still be accessible on the enzyme immobilized through its ATP binding site. This was investigated by using the immobilized 5-lipoxygenase on ATP-agarose as affinity chromatography for inhibitors of the reaction. It was necessary to perform the chromatography in the presence of CHAPS (5 mM) and NaCl (0.15 M) to reduce the nonspecific adsorption of diluted inhibitors to the gel matrix. Figure 7 shows the difference in the elution profiles of the naphthalenic lignan L-697,198 (a potent 5-lipoxygenase inhibitor) and indomethacin (a selective cyclooxygenase inhibitor), after their application on ATP-agarose containing 5-lipoxygenase. Indomethacin was exclusively recovered in the flow-through fractions. In contrast, L-697,198 was selectively retained by the immobilized enzyme with 20% of the inhibitor eluting from the column being recovered in the flow-through fractions and 80% after elution by buffer containing arachidonic acid. The subsequent elution with buffer containing ATP released the 5-lipoxygenase and no further amount of inhibitor. The 5-lipoxygenase was recovered as an inactive protein. Control experiments showed that the presence of 5-lipoxygenase is required for the retention of L-697,198. A similar binding on the immobilized 5-lipoxygenase was observed for the thiopyranoindole inhibitor L-691,816 (Hutchinson et al., 1993) with 75% of the inhibitor being recovered in the fractions following elution with arachidonic acid. These results provide further evidence for a competition between these inhibitors and arachidonic acid at a site that is accessible on the enzyme immobilized through its ATP binding site.

DISCUSSION

The present data provide evidence for the presence of an arachidonic acid binding site on 5-lipoxygenase that can be photoaffinity labeled by the thiopyranoindole L-708,714 and which is the site of interaction of a number of different inhibitors of the reaction. The conclusion that L-708,714 selectively labels the protein in the proximity of the active site is supported by the following observations: (1) L-708,714 is a potent reversible inhibitor of the reaction (IC_{50} = 0.3 μM) and exclusively labels 5-lipoxygenase after irradiation of extracts from Sf9 cells where the enzyme represents about 5% of the protein content. (2) More than 80% of the labeling can be inhibited by arachidonic acid. (3) Benzofuranols and *N*-hydroxyurea inhibitors, which function as reducing substrates for the pseudoperoxidase activity of 5-lipoxygenase (Falgueyret et al., 1992), including CPHU which has been shown to reduce the non-heme iron atom of the enzyme by EPR spectroscopy (Chasteen et al., 1993), caused a dose-dependent decrease of the photoaffinity labeling. This result suggests that the probe is binding at a site in the vicinity of the non-heme iron and is in agreement with the labeling occurring at the arachidonic binding site. This decrease in labeling appears to be due to a competition between the reducing inhibitor and the probe rather than to an indirect effect on the enzyme oxidation states since no difference in photoaffinity labeling between the oxidized and reduced forms was observed in the absence of inhibitor. (4) Thiopyranoindoles and naphthalenic lignans, which appear to compete with arachidonic acid for binding to the immobilized enzyme, were also competitors for the photoaffinity labeling of the enzyme.

In previous studies, we have shown that thiopyranoindole and lignan inhibitors can also block the oxidation of reducing agents catalyzed by 5-lipoxygenase (Falgueyret et al., 1993, Ducharme et al., 1994). The simplest interpretation for these results is that the inhibitory effects of these various non-redox and reducing inhibitors are due, at least in part, to their ability to interact with the arachidonic acid binding site of 5-lipoxygenase. The photoaffinity labeling of the enzyme was performed using trace amounts of the probe (10 nM) in order to obtain IC_{50} values that should approximate the dissociation constants for the binding of the various competing ligands. The IC_{50} value for the competition of the

photoaffinity labeling by arachidonic acid (43 μM) was slightly higher than the apparent *K*m for the reaction (IC_{50} = 8−10 μM). Similarly, several compounds were more potent (5−10-fold) at causing inhibition of 5-lipoxygenase activity than of photoaffinity labeling (Table 1). This suggests that mechanisms other than competitive inhibition could also be involved in the inhibition of the reaction or that the potency of the compounds to compete in the photoaffinity labeling experiment may be underestimated due to possible displacement in equilibrium during photolysis of the probe. The possibility that arachidonic acid and reducing inhibitors could interfere with the photoreaction by generation of free radical species cannot be completely excluded. However, the IC_{50} values for the inhibition of photolabeling are sufficiently close to either the apparent *K*m, in the case of arachidonic acid, or the IC_{50} values for the inhibition of enzymatic activity by various inhibitors to suggest there was no major interference by these compounds.

Among the co-factors reported to be required for maximal activity of 5-lipoxygenase (Percival et al., 1992), EDTA, DTT, and phosphatidylcholine did not cause any stimulation of photoaffinity labeling, suggesting that these molecules do not significantly modify the structure of the site involved in inhibitor binding. A dependence of the labeling on Ca^{2+} ions was observed only in the presence of phosphatidylcholine, in agreement with previous observations on the Ca^{2+}-dependent association of 5-lipoxygenase to phospholipid vesicles and the interfacial catalysis for the reaction (Wong & Crooke, 1991; Riendeau et al., 1993; Noguchi et al., 1994). In the presence of phosphatidylcholine, most of the hydrophobic [^{125}I]L-708,714 should partition into phosphatidylcholine vesicles and thus be dependent on Ca^{2+} ions for association of the enzyme to the vesicles such that an efficient labeling can occur.

The characteristics of the elution of 5-lipoxygenase from the ATP-agarose suggest that the stimulatory effect of ATP on enzymatic activity is mediated through a selective nucleotide binding site on the enzyme. The 5-lipoxygenase was not dissociated from the gel by an increase in ionic strength or pH (up to pH 10, data not shown) or by the presence of detergents or arachidonic acid. The elution of 5-lipoxygenase by various adenine nucleotides followed the same order of effectiveness as for the stimulation of activity (ATP > ADP > AMP). The effect of the stable ATP analogs further support the earlier conclusion that hydrolysis of the phosphodiester bond is not required for the stimulation by ATP (DeWolf, 1991). UTP, GTP, and TTP were ineffective at causing elution from the gel (20 mM) or at stimulating the activity (0.1 mM) at concentrations that were optimal for ATP. The relative effectiveness of the various nucleotides in stimulating the activity of purified human 5-lipoxygenase is identical to that reported for the partially purified enzyme from RBL cells (Furukawa et al., 1984). The 5-lipoxygenase adsorbed on ATP-agarose represent a model system of an enzyme noncovalently immobilized at an allosteric site that can be used for the affinity chromatography of active site ligands. The major drawback of this system is the instability of 5-lipoxygenase during chromatography under the conditions required to reduce the nonspecific binding of the inhibitors to the gel matrix. Nevertheless, inhibitors are selectively retained by the enzyme and eluted by arachidonic acid without dissociation of 5-lipoxygenase from the support. It seems likely that the inactivation of 5-lipoxygenase involves a localized oxidation event, similar to that of the metal-catalyzed oxidation of certain amino acids of proteins (Percival et al., 1992), which does not interfere with the binding at either site. For example, oxidatively inactivated 5-lipoxygenase binds to ATP-agarose (Percival, 1991) and reacts with the photosensitive probe in contrast to the fully denatured protein (Figure 5).

Mammalian 5-lipoxygenases show homology with soybean 15-lipoxygenase with regard to primary structure and essential amino acids for the coordination of the non-heme iron (Steczko et al., 1992; Nguyen et al., 1991; Zhang et al., 1992) but differ in substrate specificity and sensitivity to inhibitors. The three-dimensional structure of the soybean lipoxygenase has revealed the presence of a 40 Å long channel and narrow cavity which connects the non-heme iron to the exterior of the protein (Boyington et al., 1993). Most of the amino acids lining this cavity are hydrophobic and strongly conserved among the various lipoxygenases. The present data suggest that the corresponding cavity on 5-lipoxygenase is also the site of interaction of various inhibitors that have shown efficacy in the treatment of allergic asthma.

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