Stimulation of Leukotriene Production and Membrane Translocation of 5-Lipoxygenase by Cross-Linking of the IgE Receptors in RBL-2H3 Cells

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ABSTRACT: Recent studies in rat basophilic leukemia cells (RBL-2H3) have shown that two pharmacological agents, ionomycin and thapsigargin, induce leukotriene C_4 production and translocation of 5-lipoxygenase from cytosol to membrane, primarily by causing an influx of extracellular calcium. In the present study, we investigate the induction of these events by receptor activation. Cross-linking of high-affinity IgE receptors ($Fc_\epsilon RI$) by antigen in RBL-2H3 cells leads to leukotriene C_4 production and membrane translocation of 5-lipoxygenase. As in the ionomycin-stimulated cells, leukotriene C_4 production in antigen-stimulated cells is calcium-dependent since the amount of leukotriene C_4 produced correlates quantitatively with the increase in intracellular free calcium concentration ($[Ca^{2+}]_i$). However, the increase in $[Ca^{2+}]_i$ required for equivalent leukotriene C_4 production by antigen is not as high as it is using ionomycin. In addition, no threshold $[Ca^{2+}]_i$ level is required for leukotriene production by antigen, which is in contrast to the ionomycin stimulation that a $[Ca^{2+}]_i$ level of 300–400 nM is required. Furthermore, antigen causes an additive increase in leukotriene C_4 production in cells stimulated by the ionomycin. These results suggest that another as yet unidentified intracellular pathway acts in conjunction with Ca^{2+} for leukotriene synthesis in antigen-stimulated cells. Antigen stimulation causes 20–30% of the total cell 5-lipoxygenase to associate with membranes (compared with 10% in unstimulated cells) as demonstrated by enzyme activity assay and by Western Blot using antibodies to 5-lipoxygenase. Several lines of evidence support the coupling of 5-lipoxygenase translocation to leukotriene production: (1) membrane translocation of 5-lipoxygenase correlated quantitatively with leukotriene C_4 production; (2) a specific 5-lipoxygenase translocation inhibitor MK-886, which inhibits leukotriene synthesis by binding to a 5-lipoxygenase-activating protein, completely blocks the antigen-induced leukotriene C_4 production and 5-lipoxygenase translocation; (3) when cells are stimulated with antigen either in the absence of extracellular calcium or in a high potassium buffer (cell depolarization), both leukotriene C_4 production and 5-lipoxygenase translocation are completely abolished. Taken together, our data demonstrate that leukotriene production, membrane translocation of 5-lipoxygenase, and the increase in $[Ca^{2+}]_i$ are tightly coupled in antigen-stimulated cells.

Antigen is a potent stimulus for the release of cysteinyl leukotrienes in lungs of asthmatic patients. Antigen binds to immunoglobulin E (IgE)[1] which in turn interacts with the high-affinity IgE receptors ($Fc_\epsilon RI$) on the cell surface of mast cells, basophils, and eosinophils. These cells then produce cysteinyl leukotrienes which stimulate the contraction of airway smooth muscle (Dahlen et al., 1980; Hanna et al., 1981), increase microvascular permeability (Hua et al., 1985; Dahlen et al., 1981), and stimulate mucus secretion in the airway (Marom et al., 1982; Peatfield et al., 1982). The biological activity of the cysteinyl leukotrienes, together with the clinical efficacies of a 5-lipoxygenase inhibitor A-64077 (Israel et al., 1990; Knapp, 1990) and a LTD_4 antagonist MK-571 (Manning et al., 1990) in treating asthmatic patients, supports their mediator role in bronchial asthma.

Our interest is in the intracellular mechanisms involved in $Fc_\epsilon RI$-mediated leukotriene production. A convenient experimental system to study is the rat basophilic leukemia cells (RBL-2H3). This cell line appears to be of mucosal mast cell lineage and expresses $Fc_\epsilon RI$. Aggregation of $Fc_\epsilon RI$ by antigen results in a number of biochemical events culminating in the release of histamine-containing granules (Barsumain et al., 1981; Metzger, 1978). The early events include the phospholipase C-mediated hydrolysis of membrane phosphatidylinositol, a rise in the concentration of free intracellular cytosolic calcium ($[Ca^{2+}]_i$), activation of protein kinase C and guanine nucleotide binding proteins (Cunha et al., 1988; Beaven et al., 1987; Narasimhan et al., 1990), and tyrosine phosphorylation of cellular proteins (Benhamou et al., 1990).

RBL-2H3 cells contain high levels of 5-lipoxygenase (5LO), an enzyme which converts arachidonic acid to (5*S*)-5-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5HPETE) and 5,6-oxido-7,9,11,14-eicosatetraenoic acid (LTA_4). Stimulation of RBL-2H3 cells with pharmacological agents such as the calcium ionophores A23187 and ionomycin or the tumor

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[1] Abbreviations: $[Ca^{2+}]_i$, concentration of free intracellular cytosolic calcium; DNP_{25}-BSA, 25 molecules of dinitrophenol covalently linked to one molecule of BSA; EDTA, ethylenediaminetetraacetic acid; $Fc_\epsilon RI$, the high-affinity IgE receptor; FLAP, 5-lipoxygenase-activating protein; IgE, immunoglobulin E; 5LO, 5-lipoxygenase; RBL, rat basophilic leukemia; LTA_4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; LTC_4, (5*S*,6*R*)-5-hydroxy-6-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTD_4, (5*S*,6*R*)-5-hydroxy-6-cysteinyl-7,9,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; MK-886, 3-[1-(*p*-chlorobenzyl)-5-isopropyl-3-(*tert*-butylthio)indol-2-yl]-2,2-dimethylpropanoic acid.

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promoter thapsigargin results in substantial leukotriene synthesis (Wong et al., 1991). These agents cause an influx of extracellular calcium that promotes translocation of the cytosolic 5LO to membranes (Rouzer & Kargman, 1988; Wong et al., 1991). It is hypothesized that 5LO translocation is a prerequisite for enzyme activation and subsequent leukotriene production. An integral membrane protein, 5LO-activating protein (FLAP), may serve as the membrane anchor for 5LO and enable it to acquire substrate released from membrane phospholipid stores (Miller et al., 1990; Dixon et al., 1990). Despite our understanding of the mechanisms for 5LO translocation, its physiological significance still remains questionable. Early attempts to utilize physiological stimuli such as formyl-methionylleucinylphenylalanine or opsonized zymosan in stimulating human leukocytes resulted in very little leukotriene production and no 5LO translocation (Rouzer & Kargman, 1988).

The objectives of the studies described are to determine the following: (1) whether 5LO translocation occurs under physiological stimulation, specifically by $Fc_\epsilon RI$ cross-linking; (2) whether the 5LO translocation is coupled to leukotriene production; and (3) what biochemical events are involved in regulating 5LO translocation and leukotriene production. We have used Western blot analyses in conjunction with enzyme activity assay to monitor 5LO translocation. We have examined the effects of selective 5LO translocation inhibitor MK-886 (Rouzer et al., 1990) in blocking both 5LO translocation and leukotriene production. In order to test whether $Fc_\epsilon RI$ cross-linking and ionomycin utilize different intracellular pathways for leukotriene production, we have stimulated cells with a combination of the two stimuli and looked for synergistic or additive responses. We have studied the correlation between $[Ca^{2+}]_i$ changes with leukotriene production in both $Fc_\epsilon RI$- and ionomycin-stimulated cells.

Materials and Methods

Materials

The RBL-2H3 subline was kindly supplied by Dr. R. P. Siraganian (Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health). MK-886 was synthesized by the Medicinal Chemistry Department, SmithKline Beecham. Fetal calf serum was purchased from Hyclone Labs, Logan, UT. Arachidonic acid was from NuChek Prep. Inc., Elysian, MN. The radioimmunoassay kit for LTC_4 was purchased from New England Nuclear, Boston, MA. The enhanced chemiluminescence reagents were obtained from Amersham, Arlington Heights, IL. Ionomycin and fura-2 AM were obtained from Calbiochem, La Jolla, CA. 1-Alkyl-2-acetyl-phosphatidylcholine was purchased from Avanti Polar Lipids, Pelham, AL. Dinitrophenol-specific mouse monoclonal IgE was purchased from ICN Biochemicals, Costa Mesa, CA. DNP_{25}-BSA was obtained from Molecular Probes, Junction City, OR. The biotinylated goat anti-rabbit IgG and the streptavidin–(aminohexanoyl)biotin complex kit were purchased from Zymed Laboratories, San Francisco, CA. Phorbol myristate acetate and other chemicals were obtained from Sigma, St. Louis, MO. All solvents were HPLC grade and were obtained from J. T. Baker.

Methods

Preparation of Cell Cultures. The RBL-2H3 cells were maintained as monolayer cultures in 75- or 150-cm^2 flasks in Eagle's essential medium supplemented with 20% fetal calf serum as described previously (Barsumian et al., 1981). The cells were harvested by washing with Dulbecco's phosphate-buffered saline containing 1 mM EDTA, diluted to 0.3×10^6 cells/mL with culture medium, and grown in spinner culture for 24 h. To some cultures, mouse monoclonal anti-DNP IgE (100 ng/mL) was added to prime the cells to DNP–BSA.

Incubation Conditions and Subcellular Fractionation. RBL-2H3 cells were grown to a cell density of $(0.8–1.0) \times 10^6$ cells/mL. Cells were centrifuged (200*g* 10 min at 25 °C) and resuspended in 5 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose (buffer A) at 2.0×10^6 cells/mL. The cell samples (1 mL each) were stimulated by ionomycin (5 μL in ethanol) to give the desired final concentration. Cells which had been primed with IgE were added to various concentrations of DNP_{25}–BSA (0.01–100 ng/mL). Both the ionomycin and DNP_{25}–BSA were added to the microfuge tubes prior to the addition of cells. In some experiments, cells were pretreated with various concentrations of MK-886 (5 μL in ethanol) at 37 °C for 5 min. Cells were then transferred to microfuge tubes containing ionomycin or DNP_{25}–BSA. The concentrations of ethanol were maintained constant (<1%) for all samples in any given experiment. After a 20-min incubation with the ionomycin or DNP_{25}–BSA, cells were harvested by centrifugation (4 °C) at 500*g* for 10 min. The supernatants were removed and assayed for the production of LTC_4 by radioimmunoassay. The cell pellets were resuspended in homogenizing buffer (10 mM Hepes, pH 7.4, 1 mM EDTA) at $(6–7) \times 10^6$ cells/mL and incubated on ice for 20 min. The cells were lysed by vortexing vigorously two times, each for 20 s. Cell breakage was confirmed visually using a microscope. The cell lysates were centrifuged (4 °C) at 15000*g* for 30 min. The pellets were resuspended to the original volume of the cell lysates (300 μL) with the homogenizing buffer. The 15000*g* supernatant and pellet obtained are referred to as the soluble and particulate fraction, respectively. The 5LO present in the soluble and particulate fractions was determined using enzyme activity assay and Western blots.

Soluble 5LO Activity Assay. The enzyme reaction was followed spectrophotometrically (Beckman DU-7 spectrophotometer, Fullerton, CA) at room temperature by monitoring the formation of conjugated diene at 235 nm (Grossman & Zakut, 1979). An extinction coefficient of 25 000 M^{-1} cm^{-1} was used to calculate the total conversion of arachidonic acid into conjugated diene. The optimal velocity was defined as the linear portion of the reaction progress curves following the lag phase. Reaction mixtures contained 10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 μM ATP, 1.08 mM $CaCl_2$, 25 μg/mL 1-alkyl-1-acetylphosphatidylcholine (platelet-activating factor), 10 μM of arachidonic acid (5 μL in ethanol), and the soluble fraction, in a final volume of 500 μL. 1-Alkyl-2-acetylphosphatidylcholine was added together with $CaCl_2$ and ATP to stimulate 5LO activity. Other types of phosphatidylcholines such as the dioleoylphosphatidylcholine or L-α-phosphatidylcholine (type III-E, from egg yolk) could also be used (Puustinen et al., 1988; Riendeau et al., 1989). The effects of 1-alkyl-2-acetylphosphatidylcholine on 5LO kinetics will be described (Wong et al., manuscript in preparation). The reaction components were added to the cuvette in sequence: enzyme; then a mixture containing buffer, ATP, $CaCl_2$ and 1-alkyl-2-acetylphosphatidylcholine; followed by arachidonic acid. The sample was mixed by shaking it two times before placing it in the sample compartment.

5LO Protein Quantitation by Western Blot. In order to quantify the 5LO protein present in the soluble and particulate fractions, we added 33 μL of the sample buffer (8% SDS, 0.5 M Tris-HCl, pH 6.8, 40% glycerol, 5% β-mercaptoethanol,

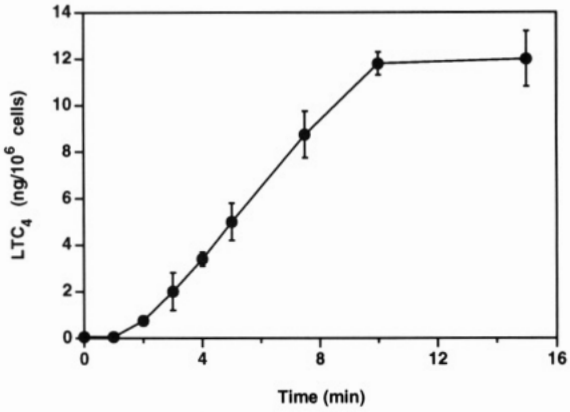

FIGURE 1: Time course of LTC_4 production induced by cross-linking $Fc_\epsilon RI$. RBL-2H3 cells were primed with a mouse monoclonal anti-DNP IgE antibody (100 ng/mL) overnight. Cells (2×10^6 cells/mL) were incubated with 30 ng/mL DNP_{25}–BSA at 37 °C. At the indicated times, two 1-mL aliquots were removed and immediately centrifuged. LTC_4 present in the extracellular media was measured by radioimmunoassay. The data show the mean ± range for LTC_4 produced by the duplicates in a representative experiment of three experiments.

and 0.003% bromphenol blue) to the 100-μL samples. The samples were incubated at 70 °C for 5 min. Aliquots (5–10 μL) of the sample mixtures were subjected to SDS–PAGE, as described by Laemmli (1970). The gels were run at 35 mA per gel for 1.5 h and were transferred to nitrocellulose paper (0.5 A for 1 h, 4 °C). The nitrocellulose sheets were blocked with a solution containing 5% nonfat dry milk in phosphate-buffered saline, containing 0.2% Tween 20. The nitrocellulose sheets were incubated with a polyclonal antiserum raised against highly purified RBL-5LO (Wong et al., 1988) diluted in blocking solution (1:500) for 1 h at room temperature. Unbound antibody was washed from nitrocellulose sheets by three changes of phosphate-buffered saline containing 0.1% Tween 20 (PBST), for 10 min each. The nitrocellulose sheets were incubated with biotinylated goat anti-rabbit IgG in milk blocking solution for 1 h and then washed three times with PBST for 10 min each. The blots were incubated with the streptavidin–(aminohexanoyl)biotin-conjugated–horseradish peroxidase complex diluted in PBST (prepared according to the manufacturer's instructions) for 1 h, then washed four times with PBST, 10 min each. The blots were immersed in the enhanced chemiluminescence reagent, drained, and immediately exposed to X-ray film. Autoradiograms were scanned with a LKB-2202 Ultroscan laser densitometer (LKB Produktor, Bromma, Sweden).

LTC_4 Radioimmunoassay. A total of 0.1 mL of the extracellular supernatants was assayed for LTC_4. Radioimmunoassays were performed in polypropylene test tubes according to the manufacturer's instructions. Synthetic LTC_4 was detectable on a linear portion of the radioligand binding curve at concentrations ranging from 0.2 to 10 ng/mL.

$[Ca^{2+}]_i$ Measurements. $[Ca^{2+}]_i$ measurements were performed as previously described (Wong et al., 1991).

Preparation of Drugs. Stock solutions of ionomycin, phorbol myristate acetate, and MK-886 were prepared in absolute ethanol and added such that the final ethanol concentration did not exceed 1% v/v in the cell suspensions. DNP_{25}–BSA was prepared in aqueous solution. Concentrations of the ionomycin and DNP_{25}–BSA stock solution were quantitated spectrophotometrically before use.

RESULTS

LTC_4 Production in Response to Cross-Linking of $Fc_\epsilon RI$ by Antigen. IgE-primed cells generated LTC_4 when incubated with antigen. At optimal antigen concentration (30 ng/mL DNP_{25}–BSA), LTC_4 production was detected within 2 min of incubation and reached its maximal level (13.4 ± 5.7 ng/10^6 cells, mean ± SEM, $n = 27$) by 10–15 min (Figure 1). Maximal amounts of LTC_4 produced by the antigen-stimulated cells varied from 8 to 20 ng/10^6 cells between experiments. Despite interexperimental variation, maximal LTC_4 produced by antigen-treated cells was always half of the amount produced by the ionomycin-stimulated cells within the same experiment. Cells primed with IgE in the absence of antigen or unprimed cells treated with antigen did not produce LTC_4.

Antigen-Induced Translocation of 5LO from Soluble to Particulate Fraction. The soluble enzyme activity of RBL-2H3 cells in the resting state was 57.0 ± 4.2 nmol/(mg·min) (mean ± SEM, $n = 15$). The majority of activity was located in this fraction. When cells were stimulated with 30 ng/mL antigen, there was a 25% ± 6% decrease in the soluble enzyme activity (mean ± SEM, $n = 15$, Figure 2a). Western blot analysis (Figure 2b,c) showed a parallel decrease in the amount of soluble 5LO protein (15.8% ± 5% of total cell 5LO, mean ± SEM, $n = 15$) and an increase of 5LO protein in the particulate fraction to 25% ± 5% of total cell 5LO compared with 10% in unstimulated cells. In the present study, the soluble fraction was defined as the 15000*g* supernatant. Previous

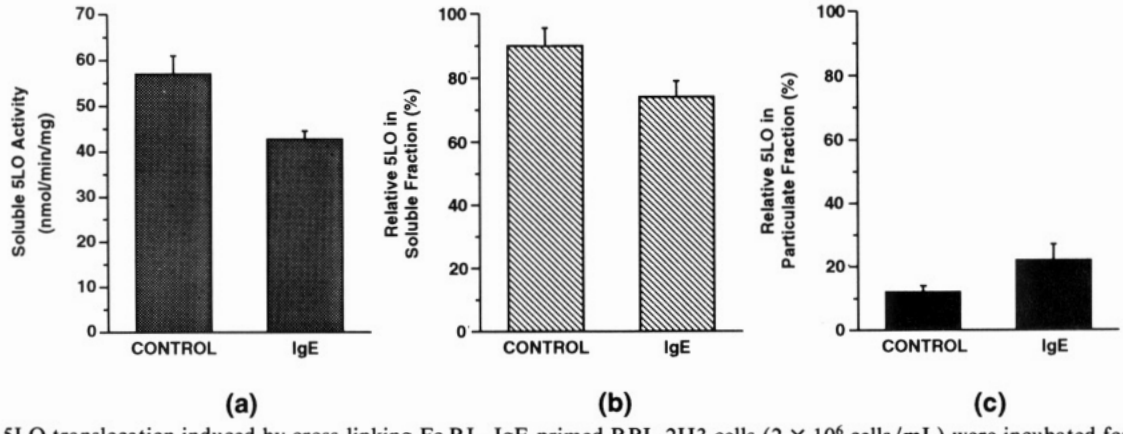

FIGURE 2: 5LO translocation induced by cross-linking $Fc_\epsilon RI$. IgE-primed RBL-2H3 cells (2×10^6 cells/mL) were incubated for 20 min at 37 °C with 30 ng/mL DNP_{25}–BSA. After incubation, cell samples were centrifuged (500*g*, 10 min). The soluble and membrane fractions were prepared and assayed as described in Materials and Methods. Panel a shows the 5LO enzyme activity in the soluble fraction determined spectrophotometrically. Panels b and c show the amounts of 5LO enzyme in the soluble and particulate fractions determined by Western blots. Total cellular 5LO enzyme (soluble plus particulate fraction) is normalized as 100%. Values represent the mean ± SEM in triplicates of five experiments ($n = 15$).

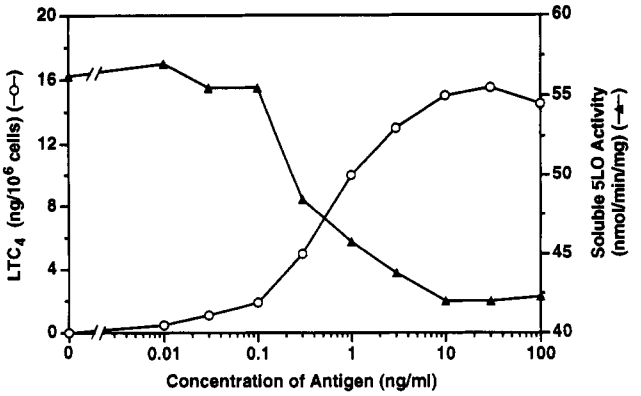

FIGURE 3: Effect of antigen concentration on LTC_4 production and soluble 5LO activity. IgE-primed RBL-2H3 cells (2×10^6 cells/mL) were incubated for 20 min at 37 °C with the indicated concentrations of DNP_{25}–BSA. LTC_4 released into the extracellular media was measured by radioimmunoassay. Cells were lysed, and the soluble fractions (15000g supernatant) were assayed for the 5LO activity. Means of duplicate samples from a representative experiment (of four experiments) are shown: (○) LTC_4 produced; (▲) soluble 5LO activity.

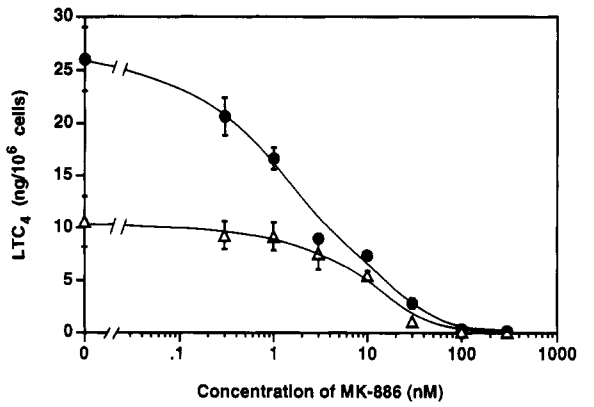

FIGURE 4: Inhibition of LTC_4 production by MK-886. RBL-2H3 cells (2×10^6 cells/mL, unprimed or IgE-primed) were incubated with the indicated concentrations of MK-886 for 5 min at 37 °C. After incubation, cells were stimulated with 150 nM ionomycin (unprimed cells) or 30 ng/mL DNP_{25}–BSA (IgE-primed cells) for 20 min at 37 °C. Extracellular media was recovered and assayed for LTC_4 using radioimmunoassay. Data shown are the mean ± SEM for triplicate samples: (●) ionomycin-stimulated cells; (△) DNP_{25}–BSA-stimulated cells. Similar experiments have been repeated three times.

studies in human leukocytes had defined the soluble fraction as the 100000g supernatant, which was microsome-free. We had compared the 15000g and 100000g supernatants in both resting and ionomycin-stimulated cells and observed no significant difference between the amounts of 5LO protein recovered in the two supernatants (data not shown). Similar 5LO activities were also recovered in the two supernatants. Therefore, separation of microsomes from the soluble fraction did not affect the distribution pattern of 5LO.

We next examined whether the antigen-induced membrane association of 5LO could be reversed by removal of antigen or by treatment with a chelator. After treatment with antigen (30 ng/mL) for 15 min, cells were harvested and resuspended in buffer A for 15 min. Cells were lysed and assayed for membrane-associated 5LO by Western blot analyses. Results indicated that the 5LO remained membrane-associated after removal of antigen (data not shown). In another experiment, cells were treated with antigen for 15 min before the addition of excess EDTA (2 mM) for 15 min. Western blots showed that EDTA did not reverse the antigen-induced 5LO translocation (data not shown). In this respect, the nature of the membrane-associated enzyme was similar to that induced by ionomycin and thapsigargin (Wong et al., 1991).

Figure 3 shows the amount of LTC_4 produced and the loss of soluble 5LO activity after antigen treatment (0.01–100 ng/mL). The two responses had similar dose–response relationships. The concentration-dependence curves are steep for decreased soluble 5LO activity and LTC_4 production at antigen concentrations of 0.1 to 3 ng/mL. Both responses reached plateau levels with antigen ≥ 10 ng/mL.

Inhibition of Antigen-Stimulated LTC_4 Production and 5LO Translocation by MK-886. In order to demonstrate that the antigen-stimulated 5LO translocation is coupled to leukotriene production, the effect of MK-886 on the antigen-induced responses is examined. MK-886 is a selective and potent inhibitor of leukotriene synthesis and 5LO translocation in human leukocytes stimulated by the calcium ionophore A23187 (Rouzer et al., 1990). In RBL-2H3 cells, MK-886 exhibited similar potencies for inhibiting the ionomycin and antigen-induced LTC_4 production (Figure 4). Half-maximal inhibition was obtained at 2 and 10 nM MK-886 in ionomycin and antigen-stimulated cells, respectively.

Parallel to the inhibition of leukotriene production, MK-886 blocked the 5LO membrane translocation induced by both

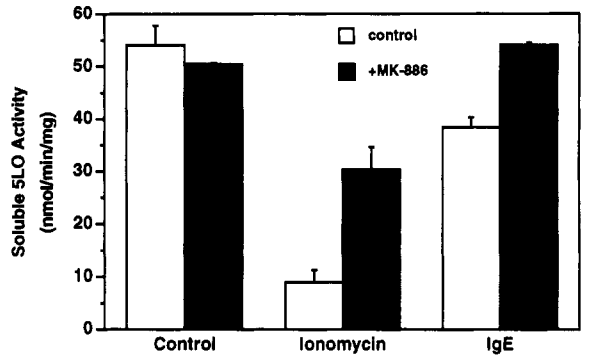

FIGURE 5: Inhibition of 5LO translocation by MK-886. RBL-2H3 cells (2×10^6 cells/mL, unprimed or IgE-primed) were incubated for 5 min at 37 °C in the presence or absence of 300 nM MK-886. Cells were then stimulated with 5 μL of ethanol (unprimed cells), 150 nM ionomycin (unprimed cells), or 30 ng/mL DNP_{25}–BSA (IgE-primed cells) for 20 min at 37 °C. Cells were lysed, and 5LO activity in the soluble fractions (15000g supernatant) was determined under standard conditions for enzyme activities. Values are mean ± SEM for triplicates in three experiments (n = 9). Solid bars and open bars represent cell samples without or with MK-886 pretreatment, respectively. Control cells are unprimed cells stimulated by ethanol.

ionomycin and antigen (Figure 5). Challenging the cells with 100 nM ionomycin resulted in an 85% decrease of soluble 5LO enzyme activity. Pretreatment of cells with an optimal concentration of MK-886 (300 nM) resulted in increased recovery of the soluble enzyme activity. The inhibition of ionomycin stimulation was not complete, and a fraction (50%) of the translocated enzyme was refractory to the MK-886 treatment. This fraction varied from 10 to 50% between experiments. The MK-886 refractory enzymes is probably the result of nonspecific enzyme aggregation caused by high $[Ca^{2+}]_i$ (≥1 μM) in ionomycin-stimulated cells. This nonspecific 5LO aggregation has been reported by other investigators (Rouzer et al., 1990). In addition, ionomycin may intercalate into membranes and induce nonspecific membrane binding of 5LO. In contrast to its effects on the ionomycin-stimulated cells, MK-886 completely blocked the membrane translocation of 5LO in the antigen-stimulated cells (Figure 5), supporting the view that 5LO translocation is coupled to leukotriene production in these cells.

Antigen Augmented LTC_4 Production Induced by Ionomycin. If antigen and ionomycin utilize different intracellular

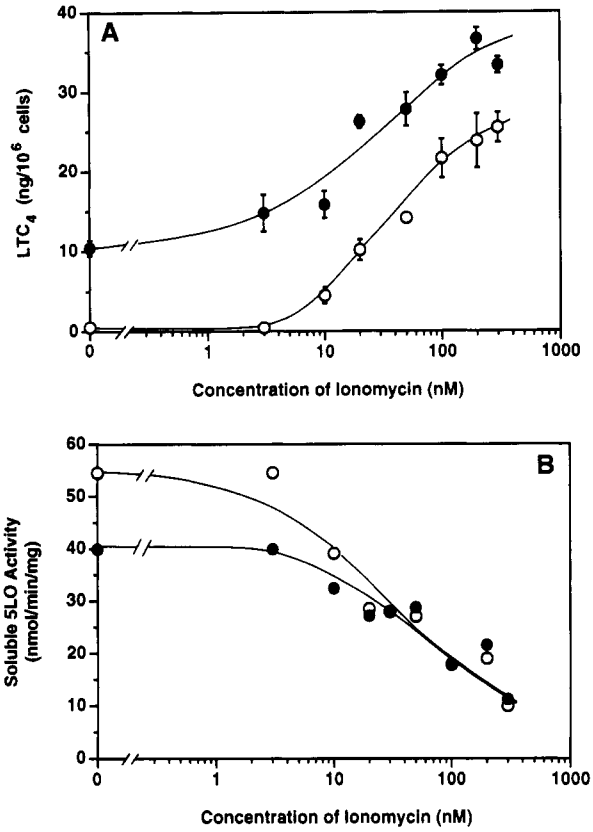

FIGURE 6: Stimulation of LTC_4 production and 5LO translocation by a combination of ionomycin and antigen. IgE-primed RBL-2H3 cells (2×10^6 cells/mL) were incubated for 20 min at 37 °C with the indicated concentrations of ionomycin in the presence (●) or absence (○) of 30 ng/mL DNP_{25}–BSA. (A) Extracellular media was analyzed by radioimmunoassay for LTC_4 production. Values presented are the mean ± SEM for triplicates in a representative experiment of total five experiments. (B) 5LO activity in the soluble fraction was assayed under standard conditions. Values are single determinations in a representative experiment of total five experiments.

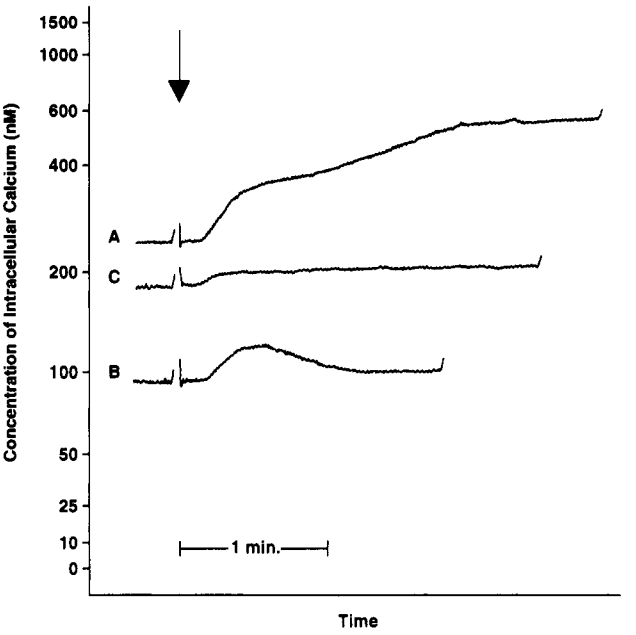

FIGURE 7: Effects of cross-linking of $Fc_\epsilon RI$ on $[Ca^{2+}]_i$. (A) Fura-2-loaded IgE-primed RBL-2H3 cells (2×10^6 cells in 1 mL of 10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 5.6 mM glucose) were equilibrated at 37 °C. DNP_{25}–BSA was added at the arrow. (B) 2 mM EGTA was added prior to the addition of DNP_{25}–BSA. (C) Fura-2-loaded RBL-2H3 cells were equilibrated in a depolarizing buffer (contained the above components, except that NaCl was completely replaced with KCl). DNP_{25}–BSA was added at the arrow. Similar results were obtained in five experiments.

mechanisms for leukotriene production, then a synergistic or additive response should be obtained when a combination of the two agonists is used in cell activation. Figure 6A shows that 30 ng/mL antigen caused additive increases in LTC_4 production in cells stimulated with ionomycin at concentrations ranging from 10 to 300 nM. Since additive responses were observed when cells were stimulated with maximal concentrations of the two agonists, different mechanisms might have been utilized for leukotriene production. Note that no cooperative action of the two agonists was observed and that the-half maximal effective concentration of ionomycin was not changed, suggesting that the responses induced by antigen and by ionomycin were highly specific or compartmentalized. In contrast, antigen did not cause an additional decrease in soluble 5LO activity at high ionomycin concentrations (>100 nM) (Figure 6B), suggesting that the two agonists might utilize a similar mechanism for 5LO translocation.

Antigen-Stimulated Increase in Intracellular Calcium Concentrations. In an attempt to identify the intracellular mechanisms for the leukotriene production induced by $Fc_\epsilon RI$ cross-linking, we have measured the $[Ca^{2+}]_i$ changes in antigen-stimulated cells, since calcium is one of the second messengers coupled to $Fc_\epsilon RI$ cross-linking. When the IgE-primed RBL-2H3 cells were stimulated with 30 ng/mL antigen, there was an increase in $[Ca^{2+}]_i$ (from 225 ± 20 nM to 510 ± 24 nM, mean ± SEM, $n = 10$) after a lag time of 6–10 s (Figure 7, tracing A). No $[Ca^{2+}]_i$ increase was observed when antigen was added in unprimed cells. After RBL-2H3 cells had been cultured for several months, the initial $[Ca^{2+}]_i$ signal in response to antigen became more prominant ($[Ca^{2+}]_i$ increased to 800 nM) whereas the steady-state $[Ca^{2+}]_i$ remained unchanged. These changes are probably due to a phenotypic drift of RBL-2H3 cells in culture as noted by other investigators (Beaven et al., 1987).

Dependence of Leukotriene Production and 5LO Translocation on Influx of Extracellular Calcium. In other experiments, an excess of EGTA (2 mM) was added to cell samples prior to the stimulation with antigen. In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ increase induced by antigen was greatly attenuated (Figure 7, tracing B). Under these conditions 5LO translocation and LTC_4 production were completely abolished.

When resuspended in a high potassium buffer, RBL-2H3 cells depolarize and become refractory to antigen stimulation to take up extracellular Ca^{2+} (Mohr & Fewtrell, 1987). In agreement with the previous report, we observed little, if any (<10%, $[Ca^{2+}]_i$ increase in response to antigen stimulation (Figure 7, tracing C). Moreover, 5LO translocation and LTC_4 production were completely absent in the depolarized cells (data not shown). Taken together, these results suggest than an influx of extracellular Ca^{2+} is essential for the antigen-induced leukotriene production and 5LO translocation.

Correlation between $[Ca^{2+}]_i$ and Leukotriene Production in Response to Antigen. Figure 8A shows the changes in $[Ca^{2+}]_i$ and the LTC_4 production at antigen concentrations of 0.01–100 ng/mL. The concentration-dependence curves are steep for both $[Ca^{2+}]_i$ increase and leukotriene production at antigen concentrations of 0.1–1 ng/mL. A half-maximal increase for both responses was obtained at 0.3 ng/mL. Plotting leukotriene production versus $[Ca^{2+}]_i$ gave a sigmoid curve, with a correlation coefficient of 0.989 (Figure 8B).

We have previously shown that a threshold $[Ca^{2+}]_i$ concentration of 300–400 nM was required for leukotriene pro-

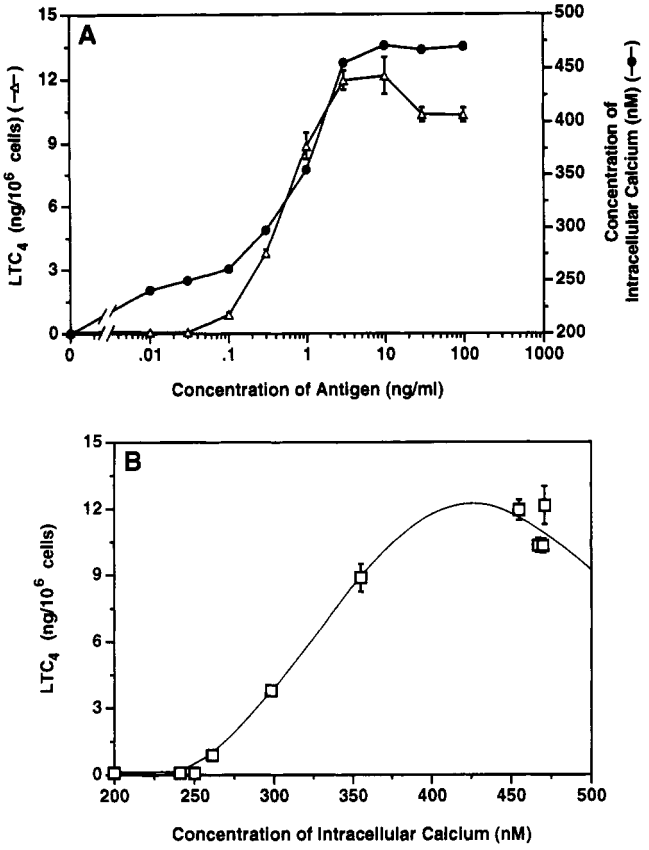

FIGURE 8: (A) Concentration dependency of antigen of $[Ca^{2+}]_i$ changes and LTC_4 production. IgE-primed RBL-2H3 cells (2×10^6 cells/mL) were incubated with the indicated concentrations of DNP_{25}–BSA for 20 min at 37 °C. LTC_4 released into the extracellular media was measured by radioimmunoassay: (●) $[Ca^{2+}]_i$ changes; (△) LTC_4 produced. Values for LTC_4 production are mean ± SEM of triplicate samples. The same cell preparation was used for both $[Ca^{2+}]_i$ and LTC_4 production measurements. (B) Quantitative relationship between $[Ca^{2+}]_i$ changes and LTC_4 production.

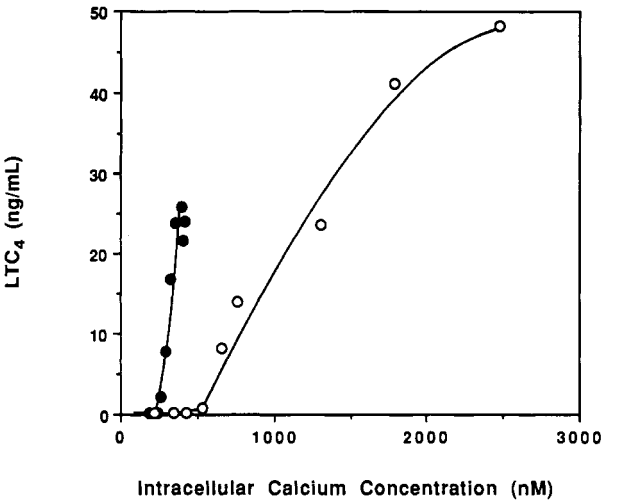

FIGURE 9: Relationship between $[Ca^{2+}]_i$ and LTC_4 production in response to antigen and ionomycin. The LTC_4 produced as a function of the stimulated $[Ca^{2+}]_i$ changes in response to 0.1–100 ng/mL DNP_{25}–BSA (●) and 10–300 nM ionomycin (○) are shown. Values are means of three experiments.

duction in both ionomycin- and thapsigargin-stimulated cells (Wong et al., 1991). In contrast, the present data suggest that there is no noticeable threshold $[Ca^{2+}]_i$ requirement for the antigen-induced response. Significant quantities of LTC_4 were obtained in response to 0.3–10 ng/mL antigen, conditions which elicit similar $[Ca^{2+}]_i$ changes as 1–10 nM ionomycin. However, ionomycin at these concentrations failed to induce LTC_4 production (Figure 9). Therefore, the relationships between $[Ca^{2+}]_i$ and leukotriene production are different for antigen- and ionomycin-stimulated cells.

Discussion

The major findings of this work are as follows. First, cross-linking of Fc$_\epsilon$RI by antigen induces leukotriene production and membrane association of 5LO. Second, the two responses are tightly coupled and are Ca^{2+}-dependent. Third, an unidentified signal acts in conjunction with Ca^{2+} for leukotriene production. The quantitative relationship between 5LO membrane association and LTC_4 production indicates coupling between the two events. Although it may seem dubious inasmuch as only 10–15% of the total cell enzyme becomes membrane-associated after Fc$_\epsilon$RI cross-linking, the coupling between the two events is strengthened in that both events are completely inhibited when extracellular Ca^{2+} is removed or when cells are depolarized. Most importantly, a selective 5LO translocation inhibitor MK-886 is able to block both LTC_4 production and 5LO translocation in the antigen-stimulated cells. The target of MK-886 has been identified as an integral membrane protein designated FLAP (Miller et al., 1990; Dixon et al., 1990). How MK-886 binding to FLAP prevents 5LO membrane association and leukotriene production is presently unknown. The current hypothesis is that 5LO activation is associated with binding of the enzyme to the docking protein FLAP. This 5LO–FLAP complex can regulate the interaction of 5LO with its substrate arachidonic acid, resulting in leukotriene production [for a review, see Ford-Hutchinson (1991)]. Since there is no proof that FLAP binds to 5LO directly, other interpretations are equally viable. One possibility is that membrane association of 5LO simply serves as a repository for inactivated enzyme since the membrane-associated enzyme obtained from the ionophore-stimulated cells is inactive (Rouzer & Kargman, 1988; Wong et al., 1988). Interestingly, after antigen stimulation we have occasionally observed an increase of membrane-associated 5LO activity. However, the extent of increase varies greatly between experiments (20–250% increase over control cells). Membrane associated 5LO activity was determined by the conversion of [^{14}C]arachidonic acid into [^{14}C]5HPETE and [^{14}C]dihydroperoxytetraenoic acid in a 4-min assay [for the method, see Wong et al. (1988)]. Because of the complicated nature of 5LO kinetics, this assay was not quantitative. At present, it is not known whether the variation was due to the 5LO assay methodology whether it was or simply because we were measuring the activity of a nonspecific aggregated enzyme. Further study is necessary to support the relevance of this observation to 5LO activation.

A second complication arising in interpreting the role of 5LO membrane association is that a linear relationship does not exist between the various stimuli in inducing 5LO membrane association and leukotriene production. The membrane-bound 5LO induced by Fc$_\epsilon$RI activation produces more LTC_4 per unit enzyme than that induced by ionomycin and thapsigargin. Since leukotriene production is subject to multistep regulation, it is possible that Fc$_\epsilon$RI cross-linking may influence leukotriene production by two sequentially operating mechanisms. The first stimulates 5LO membrane association while a second signal activates the membrane-bound 5LO or another enzyme in the leukotriene biosynthetic pathway. A recent study (Coffey et al., 1992) in rat alveolar macrophages shows that 5LO is membrane-associated in resting cells and that A23187 stimulation results in leukotriene production without causing additional membrane association of the enzyme. Therefore, the coupling between 5LO membrane

translocation, enzyme activation, and leukotriene production may be different in various cell types.

Our previous study (Wong et al., 1991) established a good correlation between $[Ca^{2+}]_i$ changes and leukotriene production in both ionomycin- and thapsigargin-stimulated cells. In the present study, we also observed good correlation between the two responses for antigen-stimulated cells. However, the curves for antigen and ionomycin activation shown in Figure 8B were not superimposable. Note that the antigen-induced Ca^{2+} response was shifted to the left and that no threshold Ca^{2+} level was required for leukotriene production. One possible explanation is that there is another as yet unidentified signal(s) which acts in conjunction with Ca^{2+} for leukotriene production. Another explanation is that localized changes in the concentration of free Ca^{2+} may be a more important factor than a uniform increase in $[Ca^{2+}]_i$ within the cells.

Although it is presently unknown what biochemical pathway is utilized by $Fc_\epsilon RI$ in conjunction with Ca^{2+} for leukotriene production, previous studies have shown that several biochemical pathways are coupled to $Fc_\epsilon RI$ cross-linking. These include the activation of protein kinase C and the guanine nucleotide binding proteins (White & Metzger, 1988; Narasimhan et al., 1990), and the tyrosine phosphorylation of cellular proteins (Benhamou et al., 1990; Yu et al., 1991). There are indications that some of these pathways may be involved in regulating leukotriene production. For instance, a role for guanine nucleotide binding proteins has been suggested by the activation of phospholipase A_2 by guanine nucleotides (Narasimhan et al., 1990). A role for protein kinase C has been suggested by the phorbol ester activation of phospholipase A_2 (Balsinde et al., 1990) and by the activity of a combination of phorbol ester and the calcium ionophore A23187 in stimulating leukotriene production (Tripp et al., 1985; Liles et al., 1987). However, we have not been able to detect any effect of phorbol myristate acetate on leukotriene production or 5LO translocation in RBL-2H3 cells.

In the present study, average $[Ca^{2+}]_i$ changes in cell populations are measured. Studies of individual RBL-2H3 cells have suggested that the temporal response to $Fc_\epsilon RI$ cross-linking is heterogeneous (Millard et al., 1988; 1989). Moreover, single cell studies using video imaging techniques have demonstrated that $[Ca^{2+}]_i$ in cells stimulated with a variety of agonists differ markedly in their temporal and spatial distribution [for a review, see Berridge (1987) and Alkon and Rasmussen (1988)]. For example, in chromaffin cells, elevation of $[Ca^{2+}]_i$ immediately beneath the plasma membrane is critical for the triggering of secretion (O'Sullivan et al., 1989). While the spatial distribution of Ca^{2+} in RBL-2H3 cells is not known, it is suggested that the plasma membranes of RBL-2H3 cells comprise a high capacity Ca^{2+} influx–efflux pathway and that local Ca^{2+} changes in the plasma membranes may be much greater than average $[Ca^{2+}]_i$ changes (Mohr & Fewtrell, 1987). Imaging measurements also show that the Ca^{2+} signal in atntigen-stimulated cells oscillates, a phenomenon which is not observed in the ionomycin-stimulated cells (Millard et al., 1988, 1989). Whether the frequency or pattern of oscillation in $[Ca^{2+}]_i$ is related to the downstream cell responses such as exocytosis and leukotriene production will require further investigation.

In summary, stimulation of LTC_4 synthesis by a physiological stimulus, such as by cross-linking $Fc_\epsilon RI$, is coupled to a small but significant increase of 5LO associated with the membranes. LTC_4 production correlates quantitatively with the changes in $[Ca^{2+}]_i$. An influx of extracellular Ca^{2+} is essential for the $[Ca^{2+}]_i$ changes, 5LO membrane association, and LTC_4 production. The intracellular mechanisms utilized by $Fc_\epsilon RI$ cross-linking in regulating leukotriene production are different from those utilized by ionomycin.

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Registry No. LTC_4, 72025-60-6; 5LO, 80619-02-9; Ca^{2+}, 7440-70-2.

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Conserved Histidine Residues in Soybean Lipoxygenase: Functional Consequences of Their Replacement†

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ABSTRACT: Sequences of 13 lipoxygenases from various plant and mammalian species, thus far reported, display a motif of 38 amino acid residues which includes 5 conserved histidines and a 6th histidine about 160 residues downstream. These residues occur at positions 494, 499, 504, 522, 531, and 690 in soybean lipoxygenase isozyme L-1. Since the participation of iron in the lipoxygenase reaction has been established and existing evidence based on Mössbauer and EXAFS spectroscopy suggests that histidines may be involved in iron binding, the effect of the above residues has been examined in soybean lipoxygenase L-1. Six singly mutated lipoxygenases have been produced in which each of the His residues has been replaced with glutamine. Two additional mutants have been constructed wherein the codons for His-494 and His-504 have been replaced by serine codons. All of the mutant lipoxygenases, which were obtained by expression in *Escherichia coli*, have mobilities identical to that of the wild-type enzyme on denaturing gel electrophoresis and respond to lipoxygenase antibodies. The mutated proteins H499Q, H504Q, H504S, and H690Q are virtually inactive, while H522Q has about 1% of the wild-type activity. H494Q, H494S, and H531Q are about 37%, 8%, and 20% as active as the wild type, respectively. His-517 is conserved in the several lipoxygenase isozymes but not in the animal isozymes. The mutant H517Q has about 33% of the wild-type activity. The inactive mutants, H499Q, H504Q, H504S, and H690Q, become insoluble when heated for 3 min at 65 °C, as does H522Q. The other mutants and the wild-type are stable under these conditions. Although the essentiality of His-499, -504, and -690 is not proven, they are tentatively considered to be prime candidates for iron ligands. Judgment on the role of H-522 is more uncertain, since mutant H522Q has weak but detectable activity. The K_m values of the active mutants and the wild-type L-1, when determined against linoleic acid, differ only moderately, indicating that His replacements do not greatly influence the binding of the substrate.

Lipoxygenases catalyze the hydroperoxidation of lipids containing one or more *cis,cis*-pentadiene moieties. All lipoxygenases thus far examined contain one atom of non-heme, non-sulfur iron per molecule. It is well established that iron participates in the reaction. When we determined the sequence of soybean lipoxygenase isozymes L-1[1] (Shibata et al., 1987) and L-2 (Shibata et al., 1988), we noted a cluster of six histidine residues in a motif of 38 amino acid residues in both enzymes and in L-3 (Yenofsky et al., 1988). Because histidine frequently participates as an iron ligand in proteins, we suggested that the histidine-rich region was involved in the binding of this metal. Histidine is a common ligand of iron in a

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[1] Abbreviations: L-1, L-2, and L-3, soybean lipoxygenase isozymes; PAGE, polyacrylamide gel electrophoresis.

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