Characterization of the Activity of Purified Recombinant Human 5-Lipoxygenase in the Absence and Presence of Leukocyte Factors*

(Received for publication, August 21, 1990)

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Purified recombinant human 5-lipoxygenase was used to investigate the catalytic properties of the protein in the presence and absence of leukocyte stimulatory factors. Recombinant human 5-lipoxygenase was purified to apparent homogeneity (95–99%) from a high expression baculovirus system by chromatography on ATP-agarose with a yield of 0.6 mg of protein per 100 ml of culture (2 × 10⁸ cells) and a specific activity of 3–6 μmol of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) per mg of protein in the presence of ATP, Ca²⁺, and phosphatidylcholine as the only factors. In the absence of leukocyte factors, the reaction catalyzed by the purified recombinant enzyme showed a half-life of maximal 5-HPETE formation of 0.5–0.7 min and was sensitive to the selective 5-lipoxygenase inhibitors BW755C (IC₅₀ = 13 μM) and L-656,224 (IC₅₀ = 0.8 μM). The reaction products of arachidonic acid oxidation were 5-HPETE and 6-trans- and 12-epi-6-trans-leukotriene B₄, the nonenzymatic hydrolysis products of leukotriene A₄ (LT₄), indicating that the purified protein expressed both the 5-oxygenase and leukotriene A₄ synthase activities (ratio 6:1). The microsomal fraction and the 60–90% ammonium sulfate precipitate fraction from sonicated human leukocytes did not increase product formation by the isolated enzyme when assayed in the presence of ATP, Ca²⁺, and phosphatidylcholine. These factors were found to stabilize 5-lipoxygenase during preincubation of the enzyme at 37°C with the assay mixture but they failed to stimulate enzymatic activity when added at the end of the preincubation period. The results demonstrate that human 5-lipoxygenase can be isolated in a catalytically active form and that protein factors from leukocytes protect against enzyme inactivation but are not essential for enzyme activity.

The 5-lipoxygenase from leukocytes is the first enzyme involved in the conversion of arachidonic acid to leukotrienes. The enzyme catalyzes both the oxygenation of arachidonic acid to 5-HPETE¹ (5-oxygenase activity) and the further conversion of 5-HPETE to unstable allylic epoxide LTA₄, (LT₄, synthase activity) (Yamamoto, 1989; Samuelson and Funk, 1989). LT₄, then serves as substrate for enzymatic hydrolysis to LTB₄ and conjugation with glutathione to yield cysteinyl-peptidoleukotrienes or can be nonenzymatically degraded to trans-LTB₄ isomers (Borgeat et al., 1985). LT₄, synthesis by 5-lipoxygenase represents the first step in the biosynthesis of leukotrienes, a group of mediators which has been implicated in the pathophysiology of inflammatory and allergic reactions (Brain and Williams, 1990; Lewis and Austen, 1984; Ford-Hutchinson, 1989).

Mammalian 5-lipoxygenases have been shown to require ATP and Ca²⁺ for activity (Yamamoto, 1989) unlike other lipoxygenases from plants and animals. The rat and human 5-lipoxygenases have been shown to translocate from the soluble to the membrane fraction during leukocyte activation (Rouzer and Kargman, 1988; Gong et al., 1988), and, recently, a membrane-bound 18-kDa protein has been identified as an essential protein for leukotriene biosynthesis (Miller et al., 1989). The cDNA for human 5-lipoxygenase has been cloned (Dixon et al., 1988; Kataoka et al., 1988) and encodes a 674-amino acid protein of molecular weight 78,000. The amino acid sequence shows 93% identity with that of rat 5-lipoxygenase and also indicates the presence of two regions of weak homology to a 17-amino acid consensus sequence of Ca²⁺-dependent membrane-binding proteins (Balcarek et al., 1988).

A hydrophobic region with homology to other lipoxygenases has been identified which contains conserved histidine residues and is postulated to represent an iron-binding domain (Shibata et al., 1988). No ATP-binding site could be predicted from the sequence information.

Procedures have been described for the purification of 5-lipoxygenase from rat basophilic leukemic cells (Goethe et al., 1985; Hogaboom et al., 1986), pig leukocytes (Ueda et al., 1986), mouse mastocytoma cells (Shimizu et al., 1986) and human leukocytes (Rouzer and Samuelsson, 1985), and more recently from bacteria and yeast expressing the human 5-

¹ The abbreviations used are: 5-HPETE, 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid; ATP-agarose, adenosine 5'-triphosphate-agarose; (5S,6R)-dihydroxy-7,9-trans,11,14-cis-eicosatetraenoic acid; 13-HOD, 13(S)-hydroxy-9-cis-11-trans-octadecadienoic acid; 13-HFD, 13(S)-hydroxy-9-cis-11-trans-octadecadienoic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; LTA₄, (5S)-trans-5,6-oxido-7,9-trans,11,14-cis-eicosatetraenoic acid; LTB₄, (5S,12R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; 6-trans-LTB₄, (5S,12R)-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid; 12-epi-6-trans-LTB₄, (5S,12S)-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid; PC, 1-α-phosphatidylcholine; P-100, 100,000 g pellet; RP-HPLC, reverse phase-high pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S-100, 100,000 g supernatant, kb, kilobase(s); bp, base pair(s).

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lipoxygenase cDNA (Noguchi et al., 1989; Nakamura et al., 1990). However, studies on purified human 5-lipoxygenase have proven to be extremely difficult because of the tediousness of the purification procedures, the low recoveries of activity (1–4%), and the extreme lability of the purified enzyme. Furthermore, previous studies have shown that the purified protein from human leukocytes had very low levels of activity unless a membrane fraction and other fractions generated during the purification procedure were added to the assay mixtures (Rouzer and Samuelsson, 1985, 1990). Thus, most of our information on the reaction catalyzed by human 5-lipoxygenase has been obtained from activity measurements in leukocyte extracts or in the presence of stimulatory fractions and inference from studies of the soybean lipoxygenase and purified 5-lipoxygenase from other sources.

In this study, we describe the isolation and characterization of recombinant human 5-lipoxygenase from a baculovirus high expression system. The purified recombinant human 5-lipoxygenase was found to be catalytically active, which has allowed for the study of the reaction catalyzed by the isolated protein and a re-examination of the effect of various protein stimulatory factors from leukocytes.

**MATERIALS AND METHODS**

**Cells and Virus—**Spodoptera frugiperda (Sf9) insect cells and Autographa californica nuclear polyhedrosis virus were obtained from C. Richardson, Biotechnology Research Institute, Montreal, Quebec. Cell counts and percent cell viability were determined in 0.2% trypan blue. Cells were cultured in Grace's complete medium (Gibco Laboratories) supplemented with 10% fetal bovine serum (Flow Laboratories Inc.), TC Yeastolate and TC lactalbumin hydrolysate (Dico Laboratories), 50 µM of gentamicin sulfate per ml and 2.5 µg of amphotericin B (Funigzone) per ml in either Falconware T flasks (Becton Dickinson Labware) or spinner flasks (Belco Glass, Inc.) at 28°C following the procedures of Summers and Smith (1987). *Echerichia coli, JM107,* was obtained from Bethesda Research Laboratories. The bacterial cells were transformed according to published methods (Hanahan, 1988).

**Chemicals and Reagents—**Restriction endonucleases were purchased from Pharmacia LKB Biotechnology Inc., and Bethesda Research Laboratories. DNA polymerase I, Klenow fragment, and T4 DNA ligase were obtained from Bethesda Research Laboratories. Radioisotopes 125I-labeled protein A (10 mCi/mg) and [α-32P]dCTP (3,000 Ci/mmol) were from Du Pont-New England Nuclear. Adenovins 5'-triphosphate-agarose (attached to beaded agarose through C-terminus) was obtained from Bethesda Research Laboratories. DNA polymerase I, Klenow fragment, and T4 ligase were obtained from Bethesda Research Laboratories.

**Construction of Recombinant Transfer Vector pJVETLZ-H5LO—**

The baculovirus expression vector pJVETLZ-H5LO was generously provided by J. Vialard and C. Richardson (Biotechnology Research Institute, Montreal, Quebec). This vector differed from the recently published vector pJViN(hv) (Vialard et al., 1990) such that the F10 promoter was replaced by the ETL promoter (Crawford and Miller, 1988) of wild type baculovirus. The vector, pGBl-H5LO, was provided by T. Nguyen, Merck Frost Centre for Therapeutic Research. A 2.1-kb EcoRI-StuI restriction fragment from pGBT-H5LO containing 11 bp of 5' noncoding, the entire coding region, and 26 bp of 3' noncoding sequence of the human 5-lipoxygenase cDNA (Dixon et al., 1988) was blunt-end-ligated into Nhel-cut pJVETLZ. Miniprep DNA from JM107 transformants was isolated, and the orientation of the inserted fragment with respect to the polyhedrin promoter was confirmed by digestion with EcoRV. The 16.1-kb vector, pJVETLZ-H5LO, was isolated from minipreps and purified on NACS columns (Bethesda Research Laboratories) and was subsequently used for co-transfection.

**DNA Transfections and Plaque Assays—**Wild type *A. californica* nuclear polyhedrosis virus DNA (1 µg) was mixed with pJVETLZ-H5LO (2 µg) and co-transfected into Sf9 cells by the calcium phosphate method (Summers and Smith, 1987). After 7 days, the cellular debris was spun down, and the supernatant was used as the source of recombinant virus for the first plaque assay. Plaque assays were carried out in culture dishes (100 × 15 mm) as previously described (Summers and Smith, 1987). The infected cell cultures were overlaid with 10 ml of 10% SeaPlaque agarose (FMC Corp., Marine Colloid Div., Rockland, ME) diluted in Grace's complete medium plus 100 µg/ml Blue-Gal (Bethesda Research Laboratories) (50 mg/ml in dimethylformamide). Blue plaques indicative of the presence of β-galactosidase activity were within 4–6 days. The plaques were picked with a Pasteur pipette and placed in 1 ml of Grace's complete medium. The virus was allowed to elute from the agarose plug overnight at 4°C. From this initial plaque assay, 12 blue plaques were subjected to dot-blot analysis (see below) and probed with a fragment of the human 5-lipoxygenase cDNA. Seven of the twelve plaques (58%) which expressed 5-lipoxygenase activity also contained the 5-lipoxygenase cDNA (data not shown). Recombinant virus from three of the seven 5-lipoxygenase positive plaques was used for the second round of plaque assays and performed with 1-, 10-, and 102-fold dilutions of virus, from which pure recombinant virus containing both 5-lipoxygenase and β-galactosidase was obtained. Usually, two to three rounds of plaque assays were sufficient in order to purify recombinant virus from wild type virus. The recombinant virus was used to infect Sf9 cells so as to produce viral stocks with titers of 105 to 108 plaque-forming units/ml. All three separate isolates of recombinant virus, under infection of Sf9 cells, produced virtually equivalent amounts of 5-lipoxygenase (data not shown). One of them, designated rvH5LO(8-1), was used in subsequent experiments.

**Nucleic Acid Dot-Blot Hybridizations—**24-Well culture plates were seeded with 5–5 × 105 cells in 1 ml of Grace's complete medium and infected with virus derived from blue plaques. Infected cells were used for the dot-blot assay according to a published method (Summers and Smith, 1987). The probe, an 813-bp *Cal-BamHI* 5-lipoxygenase cDNA fragment (Dixon et al., 1988) was labeled with [α-32P]dCTP using the Multiprim labeling system (Amersham, Canada) to specific activities of >109 dpm/µg. The dot-blot membranes were exposed to X-Omat AR film (Kodak) for 2–4 h at −70°C.

**Polyacrylamide Gel Electrophoresis and Immunoblots—**Various protein samples were prepared in electrophoresis loading buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 30% glycerol, 0.05% bromphenol blue) and were applied to 10% polyacrylamide gels according to the method of Laemmli (1970). For immunoblot analysis, proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell/Baxter, Montreal, Quebec) and probed with the rabbit anti-5-lipoxygenase LO-33 antiserum, generated against the recombinant human 5-lipoxygenase (data not shown). The nitrocellulose membrane was exposed to X-Omat AR film (Eastman Kodak Co.) with intensifying screens for 2–6 h at −70°C.

**Preparation of Lysates from Infected Cells—**Sf9 cells were grown at 27°C in 100 ml spinner cultures. The infected cell density was 1.5–2 × 106 cells/ml and infected for 44–48 h with rvH5LO(8-1). The cells were then collected by centrifugation (900 × g for 10 min, at 20°C), washed twice with Dulbecco's phosphate-buffered saline (pH 7.4) (25 ml/2 × 107 cells) and resuspended at 1.2 × 106 cells/ml in a homogenization buffer containing 50 mM potassium phosphate (pH 7.9) 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 60 µg/ml soybean trypsin inhibitor. The cells were lysed by sonication at 4°C using a Cole Parmer (4710 Series) Ultrasonic homogenizer (3 to 5 bursts of 10 s with 30-40 s, pulse mode, 70% duty cycle, and output setting at 3). The preparations were examined under the microscope to achieve effective cell lysis (ceptor) with minimal contamination. The lysate was then centrifuged at 100,000 × g for 1 h (Beckman L-65, 60 Ti rotor) at 4°C and the resulting supernatant (S-100 fraction) brought to 24 µg/ml PC by the addition of a 250-fold concentrated solution in ethanol. The S-100 fraction (1-3 mg/ml) was stable for several hours at 4°C and could be stored for several months at −70°C in 20% ethylene glycol with about 50% recovery of activity.

**Affinity Purification of Recombinant Human 5-Lipoxygenase—**A chromatography column (0.7 × 15 cm) containing 2 ml of ATP-agarose was equilibrated with 14°C in a buffer containing 50 mM potassium phosphate (pH 7.3), containing 1 mM EDTA, 1 mM dithiothreitol, and 24 µg/ml PC. The S-100 fraction (8 ml) was applied at a flow rate of 5 ml/h, and the column was washed at a flow rate of 10 ml/h with 2 ml of the equilibration buffer. After a high salt wash (10 ml of 0.5 M NaCl...
in equilibration buffer), the column was re-equilibrated with 5 ml of buffer before elution of 5-lipoxygenase with 20 mM ATP in equilibration buffer (Wiseman, 1989). All buffers were filtered through 0.22-

μm membranes (Millex-GS, nonsterile, Millipore) and purged with helium gas. Fractions containing 5-lipoxygenase activity were identified using the spectrophotometric assay and pooled. The enzyme could be stored as aliquots in 20% ethylene glycol at -70°C for several months with approximately 50% recovery of activity.

**RP-HPLC Assay for 5-Lipoxygenase Activity**—The enzyme reaction was performed in 0.025 M Na+/-K+-phosphate buffer (pH 7.3) containing 0.5 mM CaCl2, 1 mM ATP, 24 μg/ml PC, and 20 μM arachidonic acid in a final volume of 200 μl. Arachidonic acid was added as a 2 mM solution in ethanol prior to the initiation of the reaction with the addition of enzyme. After an incubation of 5 min at room temperature, the reaction was stopped by mixing with 0.8 ml of diethyl ether/methanol/1 M citric acid (30:4:1) containing 100 ng of 13-HOD as internal standard. The mixture was centrifuged at 1,000 × g for 5 min. The upper phase was collected and dried under N2, and the residue was re-dissolved in 100 μl of the RP-HPLC solvent system. This sample was injected on a Nova-Pak C18 column (Waters) eluted isocratically with 0.1% acetonitrile/water/acetic acid (65:35:0.1) at 2 ml/min. The effluent was monitored at 235 nm for the detection of 5-HETE and 5-HPETE which eluted at 3.0 and 3.4 min, respectively. These products were quantitated from an absorbance area relative to the 13-HOD standard and were corrected for a low level of background using control samples incubated in the absence of enzyme or arachidonic acid. The reaction products were 5-HPETE for the purified enzyme and a mixture of 5-HPETE and 5-HETE for assays of the S-100 fraction or for the purified enzyme in the presence of protein factors. The 5-lipoxygenase activity is expressed as the maximal amount of 5-(H)P(ET)E accumulated after incubation (Rouzer and Samuelsson, 1985) under conditions where arachidonic acid is not limiting (<40% conversion). The amount of protein for the calculation of specific activity was measured using the Bio-Rad dye-binding assay (Bradford, 1976) and bovine serum albumin as standard.

**Spectrophotometric Assay of 5-Lipoxygenase Activity**—The kinetics of 5-HPETE production was measured from the increase in A234nm upon incubation of the purified enzyme with arachidonic acid, as previously described for the assay of porcine 5-lipoxygenase (Riedeau et al., 1989a). The reaction was measured at room temperature in 0.05 M sodium phosphate buffer (pH 7.4), 0.4 mM CaCl2, 24 μg/ml PC, 20 μM arachidonic acid and was initiated by the addition of enzyme. ATP concentrations were adjusted to 0.2 mM taking into account the ATP concentrations were adjusted to 0.2 mM taking into account the 

maximal rate of conjugated 5-HETE production was measured from the increase in A235 nm upon incubation of the enzyme and a mixture of 5-HPETE and 5-HETE for assays of the S-100 fraction or for the purified enzyme in the presence of protein factors. The 5-lipoxygenase activity is expressed as the maximal amount of 5-(H)P(ET)E accumulated after incubation (Rouzer and Samuelsson, 1985) under conditions where arachidonic acid is not limiting (<40% conversion). The amount of protein for the calculation of specific activity was measured using the Bio-Rad dye-binding assay (Bradford, 1976) and bovine serum albumin as standard.

**Resolution of the Reaction Products of 5-Lipoxygenase and Measurement of LTA4, Synthase Activity**—The reactions were performed using purified recombinant human 5-lipoxygenase under the conditions described for the spectrophotometric assay. The reaction (200 μl) was stopped after a 4-min incubation by the addition of 400 μl of diethyl ether/methanol/1 M citric acid (30:4:1). The upper phase was removed and dried under N2, and the residue was dissolved in 100 μl of methanol. NaBH4 (0.01%) was added (1 μl of a 1% solution in methanol), and the mixture was brought to 0.04% acetic acid after 2 min. Samples were then injected onto a Nova-Pak C18 column at a flow rate of 1 ml/min using methanol/water/acetic acid (75:25:0.91) as eluting solvent (Ueda et al., 1986). This chromatographic system resolves 6-trans-LTB4, 12-epi-6-trans-LTB4, LTB4, (5S,6R)-di-HETE, and 5-HETE with retention times of 5.0, 5.5, 5.9, 10.7, and 23.2 min, respectively. The elution of 5-HETE was followed by monitoring absorbance at 235 nm and that of the trienes at 270 nm.

**RESULTS**

**Expression of Human 5-Lipoxygenase cDNA in Insect Cells**—Time course analysis of human 5-lipoxygenase expression in insect Sf9 cells after infection with the recombinant virus rH5LO(8-1) was undertaken to optimize conditions for the production of soluble and active enzyme. At various time points over the course of the 96 h of infection, aliquots were removed and analyzed by SDS-PAGE and immunoblot. A 75-kDa protein, expressed in infected Sf9 cells, was readily detected on a Coomassie Blue-stained gel 40 h postinfection (Fig. 1) and was present as early as 24 h as detected by immunoblot using antisera generated against the purified human 5-lipoxygenase (see Fig. 2A), thus confirming the high level expression of recombinant 5-lipoxygenase in insect cells. This protein band could not be detected by SDS-PAGE (Fig. 1) or immunoblot (data not shown) in uninfected Sf9 cells or cells infected with wild type baculovirus. The production of 5-lipoxygenase reached maximal levels by 44–48 h, constituting at least 10% of the total cellular protein content.

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**Fig. 1.** Coomassie Blue-stained SDS-PAGE of proteins produced in uninfected Sf9 cells, Sf9 cells infected with wild type baculovirus, or rH5LO(8-1). Total proteins from 3 × 10^6 cells were solubilized by lysing the cells in the electrophoresis sample buffer at 24, 48, 40, 44, 56, 65, 72, 89, and 96 h postinfection and resolved by SDS-PAGE. The positions of protein standards (Kd) and of recombinant human 5-lipoxygenase (rH5LO) are indicated. The polyhedrin protein (34 kDa) is readily visible in the sample from wild type baculovirus infected cells.

**Fig. 2.** A, autoradiogram of an immunoblot of 5-lipoxygenase produced in Sf9 cells infected with recombinant virus rH5LO(8-1). The S-100 (S) and P-100 (P) fractions were prepared from 1.8 × 10^6 cells at 24 h or 0.9 × 10^6 cells at 40, 48, 56, 72, and 96 h postinfection. The proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and successively incubated with the LO-33 antiserum (1/300 dilution) and 125I-protein A as described under “Materials and Methods.” The immunoblot was exposed to x-ray film with intensifying screens for either 25 h (24-h samples) or 2 h (other samples) at -70°C. B, time course of 5-lipoxygenase activity and of cell viability of Sf9 cells infected with recombinant virus rH5LO(8-1). The percentage of viable cells (●) and the level of 5-lipoxygenase activity in the S-100 fraction (●) were determined using aliquots from a 100-ml culture removed at the indicated times postinfection.
In order to determine the proportion of 5-lipoxygenase in the soluble fraction, S-100 and P-100 fractions from sonicated cells were analyzed by immunoblot (Fig. 2A). Maximal level of 5-lipoxygenase was reached in the S-100 fraction by 48 h, with about half of the amount of the protein also found in the P-100 fraction at this time. The level of soluble 5-lipoxygenase then decreased with time such that, by 96 h, greater than 90% of the protein resided in the P-100. These data correlated well with the activity profile of the soluble fractions, showing a peak at 44–48 h (Fig. 2B) where high specific activities (300–600 nmol of 5-H(PE)TE/mg of protein) were observed. The activity decreased rapidly by 56 h, along with cell viability (50%) and the redistribution of 5-lipoxygenase from the S-100 to the P-100 fraction. The P-100 fraction contained barely detectable levels of 5-lipoxygenase activity. Therefore, in order to maximize the amount of soluble 5-lipoxygenase that could be purified from synchronously infected Sf9 cells, the cultures were processed 48 h postinfection.

**Affinity Purification of Recombinant Human 5-Lipoxygenase by ATP-Agarase Chromatography**—The enzyme from the infected cell extracts was purified by chromatography on ATP-agarase according to a procedure similar to that described for the partial purification of rat 5-lipoxygenase (Wiseman, 1989) with the addition of PC to the buffers to stabilize the enzyme. The elution profile of Fig. 3 shows that 5-lipoxygenase can be selectively adsorbed onto this gel with most of the proteins eluting in the flow-through or after a high salt (0.5 M NaCl) wash of the column. The 5-lipoxygenase was eluted using a buffer containing 20 mM ATP with recoveries of activity of 30–50% and specific activities for different enzyme preparations ranging from 3 to 6 pmol of 5-HPETE per mg of protein. From specific activity values and a molecular weight of 78,000 for the enzyme, it can be calculated that the 5-lipoxygenase reaction would undergo a maximum of about 500 turnovers before enzyme inactivation. Analysis of the various fractions by SDS-PAGE shows that the elution of enzyme activity with ATP coincided with that of a highly purified 75-kDa protein as revealed by Coomassie Blue staining (Fig. 4A) and immunoblot analysis with the antiserum against the leukocyte 5-lipoxygenase (Fig. 4B). About 0.6 mg of purified 5-lipoxygenase could be recovered from 100 ml of infected cell culture (2 × 10^6 cells).

The migration of the purified enzyme from insect cells on SDS-PAGE was identical with that of the 5-lipoxygenase as determined by Coomassie Blue staining (Fig. 4A) and assay of 5-lipoxygenase activity (Fig. 4B). The proteins from the elution fractions obtained after ATP-agarose chromatography were resolved by SDS-PAGE and the gels stained with Coomassie Blue or analyzed for the 5-lipoxygenase protein by immunoblotting using the antibody against the leukocyte 5-lipoxygenase (Fig. 4B). The individual fractions were, from left to right and with the number in parentheses, corresponding to the elution fractions of the profile depicted in Fig. 3: S-100 fraction, flow-through fractions (3, 11), salt wash (14, 17, 20), and the fractions eluting with 20 mM ATP (29–33).

**Fig. 3. Chromatography of recombinant human 5-lipoxygenase on ATP-agarose.** The S-100 fraction (11 mg of protein) from a 100-ml culture of infected Sf9 cells was applied on an ATP-agarose column. The column was washed with buffers and 0.5 M NaCl before elution with 20 mM ATP as described under "Materials and Methods." Fractions of 0.83 ml were collected and analyzed for protein content (■) and assay of 5-lipoxygenase (bar graph), reported as the total activity (nanomoles of 5-HPETE/5 min) of each fraction.

**Fig. 4. SDS-PAGE analysis of the ATP-agarose chromatography elution profile.** The proteins from the S-100 fraction and the elution fractions obtained after ATP-agarose chromatography (10 μl) were resolved by SDS-PAGE and the gels stained with Coomassie Blue (A) or analyzed for the 5-lipoxygenase protein by immunoblotting using the anti-5-lipoxygenase LO-33 antiserum (1/500) and labeling with 125I-protein A (B). The individual fractions were, from left to right and with the number in parentheses, corresponding to the elution fractions of the profile depicted in Fig. 3: S-100 fraction, flow-through fractions (3, 11), salt wash (14, 17, 20), and the fractions eluting with 20 mM ATP (29–33).

**Fig. 5. Comparison of purified 5-lipoxygenases from insect cells and human leukocytes.** Human 5-lipoxygenase was purified either from the S-100 fraction (Bac. S-100) of Sf9 cells infected with rH5LO(8-1) using affinity chromatography (Bac. 5-LO) or from peripheral blood leukocytes using the procedure of Rouzer and Samuelsson (1985) (Leuk. 5-LO). The proteins were analyzed by SDS-PAGE and visualized either by Coomassie Blue staining or autoradiography of the immunoblot proteins using a 1/500 dilution of the LO-33 antiserum.

isolated from human leukocytes according to the procedure described by Rouzer and Samuelsson (1985). These results are shown in Fig. 5, using both the Coomassie Blue staining and immunoblotting techniques to identify the protein. Various enzyme preparations were estimated to be 95 to 99%
homogenous after electrophoresis by scanning densitometry of the stained gel.

Kinetics of Arachidonic Acid Oxidation by Purified Human 5-Lipoxygenase—The kinetics of 5-HPETE production by the purified 5-lipoxygenase was followed spectrophotometrically from the increase in A_{234} (conjugated diene) upon incubation of the enzyme with arachidonic acid, ATP, Ca^{2+}, and PC (Fig. 6). The reaction progress curve shows the typical profile for 5-lipoxygenase with a rapid apparent first order decay in activity (t<sub>1/2</sub> = 0.6-0.7 min) and maximal product formation being reached by 3 min, before complete substrate consumption. Removal of Ca^{2+} ions from the incubation caused a very large decrease in the optimal rate of the reaction, which was attained only after a long (>1 min) lag phase (Fig. 6). Calcium concentrations had to be >0.1 mM for maximal activity (data not shown). Less than 10% activity was observed in the absence of PC (data not shown), and no activity could be detected with the omission of both Ca^{2+} and PC (Fig. 6). The reaction was inhibited by the selective 5-lipoxygenase inhibitors L-656,224 and BW755C, with a short lag phase being also observed during inhibition by BW755C (Fig. 6). The IC<sub>50</sub> values for the inhibition of the purified human 5-lipoxygenase by L-656,224 and BW755C were 0.8 μM and 13 μM, respectively.

The optimal velocity of the reaction, as estimated from the initial rate of conjugated diene formation by spectrophotometry, varied linearly with enzyme concentration (0.2-5 μg/ml) (data not shown). The specific activity of the freshly purified enzyme using this assay was 3-6 pmol of 5-HPETE/min/mg of protein. RP-HPLC analysis of the reaction mixture after incubation indicated that 5-HPETE is the predominant product that absorbs at 234 nm, with only traces of 5-HETE (not shown).

The reaction products of the 5-lipoxygenase reaction were resolved by RP-HPLC to determine whether the purified enzyme also possesses LTA₄ synthase activity in the absence of protein stimulatory factors. Fig. 7 shows the chromatogram of the 5-lipoxygenase reaction products obtained after sodium borohydride reduction and as monitored by absorbance at 235 and 270 nm. Under these conditions, 5-HPETE is reduced to 5-HETE which was detected as a major peak eluting at the position of the synthetic 5-HETE standard (23 min) and having an absorption maximum at 235 nm (data not shown). Two other peaks which had a higher absorption at 270 nm were found to elute at the positions of 6-trans-LTB₄ and 12-epi-6-trans-LTB₄. These peaks were about equal in intensity and showed the UV spectra characteristic of that of trienes (not shown) and correspond to the known nonenzymatic hydrolysis products of LTA₄. Other very minor peaks were not identified; one of them eluted at the position of (5S,6R)-diHETE and could have arisen from the 6R-oxygenase activity of the enzyme, as demonstrated for the porcine 5-lipoxygenase (Ueda and Yamamoto, 1988). The ratio of the products from the 5-lipoxygenase activity (5-HETE from 5-HPETE) to that of the LTA₄ synthase activity (trans-LTB₄, isomers) was about 6:1.

Effect of Protein Factors from Human Leukocytes on the Activity of Purified 5-Lipoxygenase—The high recovery of 5-lipoxygenase activity after the rapid affinity purification raises questions on the requirement of the various protein factors from human leukocytes that have been reported to stimulate enzyme activity (Rouzer and Samuelsson, 1985). Two of the factors were the microsomal (P-100) pellet and the proteins precipitating at 60-90% saturation of ammonium sulfate (60-90% precipitate). A stimulation of the activity of the purified recombinant enzyme by these factors could be observed using identical amounts of protein factors and assay conditions to those described by Rouzer et al. (1988a) and Rouzer and Kargman (1988). These data are summarized in Table I (assay condition 1). Each of the 60-90% precipitate or P-100 factors caused a significant increase in 5-lipoxygenase activity with a maximal 12-fold stimulation being observed in the presence of both factors. However, this higher level of specific activity could also be measured in the absence of protein factors under the standard assay conditions used in the present work (assay condition 2), which are somewhat milder than those used for the leukocyte enzyme (no prein-

Fig. 6. Kinetics and inhibition of the 5-lipoxygenase reaction. The activity of purified recombinant 5-lipoxygenase (2.3 μg/ml) was measured under the conditions described for the spectrophotometric assay (●), in the presence of 0.4 μM L-656,224 (■), 5 μM BW755C (○), or in the absence of calcium (◇) and of calcium and PC (◇).
been possible to isolate the enzyme in a catalytically active purification of recombinant human 5-lipoxygenase, it has form and further investigate the effects of leukocyte factors protection of the enzyme against rapid loss of activity during to 8% using a preincubation time of 5 min (Table al.).

Additional experiments showed that neither of the 60-90% precipitate nor the mixture 60-90% precipitate could be due to a nonspecific binding of arachidonic acid and 13-HPOD in ethanol. The incubation mixture for assay condition 2 contained 20 μM arachidonic acid, 1 mM ATP, 0.5 mM CaCl2, 24 μg/ml PC, 4.8 μM/ml affinity-purified 5-lipoxygenase, and the same amounts of protein factors as under condition 1 when included. The reaction time was 5 min at room temperature, and no preincubation was used. Protein factors contained no appreciable amount of activity when tested individually. The specific activity is reported as nmol of 5-H(P)ETE formed per mg of protein at the end of the incubation period (means ± S.E.), ND, not detectable.

| Assay conditions | Protein factors added | Specific activity nmol/mg of protein |
|------------------|-----------------------|-----------------------------------|
| Condition 1      | P-100                 | 91 ± 30 (n = 4)                   |
| Condition 1      | 60-90% precipitate    | 520 ± 35 (n = 7)                  |
| Condition 1      | P-100 + 60-90% precipitate | 932 ± 21 (n = 3)               |
| Condition 1      | 60-90% precipitate    | 1070 ± 20 (n = 3)                 |
| Condition 2      | P-100 + 60-90% precipitate | 1180 ± 40 (n = 4)               |
| Condition 2      | ND                    | ND (n = 4)                        |
| Condition 2 at 37°C | P-100 + 60-90% precipitate | 1840 ± 300 (n = 4)             |
| Condition 2 at 37°C | ND                    | ND (n = 2)                        |

**DISCUSSION**

Using improved procedures for the expression and affinity purification of recombinant human 5-lipoxygenase, it has been possible to isolate the enzyme in a catalytically active form and further investigate the effects of leukocyte factors on enzyme activity. The expression of high levels of soluble enzyme was achieved in Sf9 cells infected with recombinant baculovirus carrying the cDNA for human 5-lipoxygenase (Dixon et al., 1988) downstream of the strong polyhedrin promoter. The production of 5-lipoxygenase in the S-100 fraction from Sf9 cells infected with rH5L0(8-1) (300-600 nmol of 5-HPETE/mg of protein) represents a 10-fold improvement in yield over the previously described preparation from insect cells (Funk et al., 1989). The reasons for this increase are not clear since the transfer vectors used in both systems were essentially identical in the nucleotide sequences spanning the polyhedrin start codon. One difference was that the 5-lipoxygenase cDNA used by Funk et al. (1989) contained 34 bp of 5'-noncoding and 162 bp of 3'-noncoding sequences versus only 11 and 85 bp of 5'-noncoding and 3'-noncoding sequences, respectively, used here. Perhaps translational efficiency was suboptimal since the amount of expressed protein was lower than would be expected from the abundance of the 5-lipoxygenase mRNA they detected (Funk et al., 1989). It is possible that the removal of most of the GC-rich 5'-noncoding region of the cDNA contributed to the increase in translated protein. Differences in mRNA structure have been shown to affect translational efficiency (Kozak, 1983, and references therein). Human 5-lipoxygenase was purified by a single chromatography step on ATP-agarose to a specific activity of 3-6 μmol of 5-HPETE/mg of protein, a value in the range of, or slightly higher than, those reported for the purified human enzyme expressed in E. coli (Noguchi et al., 1989), yeast (Nakamura et al., 1990), and other mammalian 5-lipoxygenase (Yasamoto, 1989), with 30-50% recovery of activity. The latter value is significantly higher than those of 1.5% and 1% obtained for the recombinant enzymes from bacterial and yeast extracts, respectively, and of that of 4.4% for the human enzyme purified from leukocytes (Rouzer and Samuelsson, 1985) which required several chromatography steps.

The adsorption of human 5-lipoxygenase on ATP-agarose and its selective elution with ATP but not with high salt concentrations, as reported for the rat enzyme (Wiseman, 1989), suggests that adsorption on ATP-agarose occurs through a specific ATP-binding site, presumably at the site involved in the stimulation of enzyme activity by ATP. The localization of this site, as well as the mechanism by which ATP stimulates the reaction, remains to be characterized since sequence comparisons have failed to reveal any homology between 5-lipoxygenases and ATP-binding domains of other proteins (Dixon et al., 1988; Matsumoto et al., 1988).
HPETE product (Ueda and Yamamoto, 1988).

It has been reported that 5-lipoxygenase, after purification from human leukocytes, requires the presence of other protein stimulatory factors from leukocyte extracts for maximal activity. However, the present values for the unstimulated enzyme are in the range of those reported for the purified leukocyte enzyme stimulated by protein factors (3 μmol/mg of protein, Rouzer et al., 1988a; 12 μmol/mg of protein, Rouzer and Samuelsson, 1985). (The latter high number may suggest that a certain amount of inactive protein may be present in most enzyme preparations.) Three stimulatory fractions were initially resolved: the microsomal pellet (P-100), the 60–90% ammonium sulfate precipitate (60–90% precipitate), and a flow-through fraction from anion exchange chromatography (Rouzer and Samuelsson, 1985), the latter fraction being optional depending on the purification scheme (Rouzer and Samuelsson, 1990). From the results with the recombinant human 5-lipoxygenase, it can be concluded that the P-100 fraction and the 60–90% precipitate are not required for high activity, but that they do protect the enzyme against rapid loss of activity after dilution with the assay mixture under certain conditions. However, these factors have been shown to stimulate the kinetics of the reaction in crude preparations where the enzyme is not as labile (Rouzer et al., 1988b). The effect of the P-100 fraction on enzyme activity in crude preparations has been shown to be mimicked by replacement with PC and other membrane lipids (Puustinen et al., 1988), although we have observed that PC was not as efficient as the P-100 fraction in stabilizing the purified enzyme during preincubation in the assay mixture (data not shown).

The demonstration that human 5-lipoxygenase is catalytically active in the absence of leukocyte factors suggests that the human enzyme is more similar to other mammalian 5-lipoxygenases than originally thought, in agreement with recent data that revealed a 93% identity in the amino acid sequences of the rat and human 5-lipoxygenases (Balcarek et al., 1988). However, it is possible that some other factors from leukocytes could stabilize the 5-lipoxygenase and stimulate the activity under certain specific conditions. Intuitively, one could expect that the activity of such an unstable and self-inactivating enzyme might be regulated by other components. Another unidentified protein, purified from rat leukocyte extracts by gel filtration, has been shown to replace the hydroperoxide activation of 5-lipoxygenase (Riendeau et al., 1989). Recent results have also demonstrated the presence of an 18-kDa protein from human and rat leukocytes which can modulate leukotriene biosynthesis in intact cells (Miller et al., 1990; Dixon et al., 1990). Clearly, there is still much to be learned about the effects and the role of these various proteins and which assay conditions are the most representative of the cellular and in vivo situations.

The 5-lipoxygenase is the first enzyme of the leukotriene biosynthesis pathway and as such is an important potential drug target (Fitzsimmons and Rokach, 1989). Using the present procedure, about 0.6 μg of recombinant 5-lipoxygenase has been purified from a 100-ml suspension culture of infected Sf9 cells, this yield being at least 50-fold higher than those reported for recombinant enzymes from E. coli (90 μg/liter) (Noguchi et al., 1989) and yeast cultures (50 μg/liter) (Nakamura et al., 1990). The ability to produce and readily purify large amounts of active recombinant human 5-lipoxygenase should greatly facilitate not only studies aimed at elucidating the catalytic and structural properties of the enzyme but also will allow for more detailed evaluation of novel potential inhibitors of 5-lipoxygenase to modulate leukotriene biosynthesis in human diseases.

**Acknowledgments**—We would like to thank M. Bannville, J. Viard, and C. Richardson for their invaluable help and advice concerning baculovirus expression systems as well as for the transfer vector, pJVETLZ. We are also grateful to J. Evans and C. Rouzer for the purified 5-lipoxygenase from human leukocytes, G. Reid for the titer analysis of the LO-33 antiserum, and S. Kargman for the preparation of leukocyte factors. We wish to thank M. Gresser and C. Picket for their support and critical reading of the manuscript.

**REFERENCES**

Balcarek, J. M., Theisen, T. W., Cook, M. N., Varrichio, A., Hwang, S.-M., Strohsacker, M. W., and Crooke, S. T. (1988) *J. Biol. Chem.* **263,** 13937–13941

Belanger, P., Maycock, A., Guindon, Y., Bach, T., Dollob, A. L., Dufresne, C., Ford-Hutchinson, A. W., Gale, P. H.,Hopple, S., Lau, C. K., Lets, L. G., Luell, S.,McFarland, C. S., MacIntyre, E., Meurer, R., Miller, D. K., Piechuta, H., Riendeau, D., Sokol, J., Rouzer, C., and Scheingart, J. (1987) *Can. J. Physiol. Pharmacol.* **65,** 2441–2448

Borgeat, P., Nadeau, M., Salari, H., Poubelle, P., and Frutéau de Lacroix, R. (1986) *Adv. Lipid Res.* **21,** 47–77

Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254

Brain, S. D., and Williams, T. J. (1990) *Pharmacol. Ther.* **46,** 57–66

Crawford, A. M., and Miller, L. K. (1988) *J. Virol.* **62,** 2773–2781

Dixon, R. A. F., Jones, R. E., Diehl, R. E., Bennett, C. D., Kargman, S., and Rouzer, C. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 416–420

Dixon, R. A. F., Diehl, R. E., Opas, E., Randis, E., Vickers, P. J.,...
