Cellular Oxygenation of 12-Hydroxyeicosatetraenoic Acid and 15-Hydroxyeicosatetraenoic Acid by 5-Lipoxygenase Is Stimulated by 5-Lipoxygenase-activating Protein*

(Received for publication, June 2, 1998, and in revised form, August 13, 1998)

Joseph A. Mancini‡, Heather Waterman, and Denis Riedeau

From the Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec H9R 4P8, Canada

It has been proposed that 5-lipoxygenase (5-LO)-activating protein (FLAP) is an arachidonate transfer protein for leukotriene biosynthesis. Using the Spodoptera frugiperda (Sf9) insect cells, we demonstrate that FLAP causes a large stimulation (190-fold) of the conversion of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) to 5,12-diHETE when co-expressed with 5-lipoxygenase. We also demonstrate that FLAP can stimulate (2–2.5-fold) the oxygenation of 15(S)-HETE by 5-LO to 5,15-diHETE. The stimulation of both 12(S)-HETE and 15(S)-HETE oxygenation by 5-LO is completely inhabitable by the FLAP inhibitor, MK-886. In order to determine which residues of FLAP are important for 12(S)-HETE and arachidonic acid utilization by 5-LO, various mutants of FLAP were co-expressed with 5-LO in Sf9 cells. The FLAP deletion mutants del 37–53, del 52–58, del 106–108, and del 148–161 and the point mutant D62N were analyzed. The D62N mutation, which reduces the binding of indole inhibitors to FLAP, had no effect on the stimulation of substrate utilization by 5-LO. In contrast to wild type FLAP, the mutant proteins del 37–53, del 106–108, and del 148–161 failed to stimulate 12(S)-HETE and arachidonic acid utilization by 5-LO. Only one of the latter three mutations (del 37–53) has been shown to abolish the binding of indole inhibitors to FLAP. These results suggest that the lipid binding site of FLAP overlaps the inhibitor binding site and occupies several regions of the protein not essential for inhibitor binding. Because FLAP can stimulate the utilization of 12(S)-HETE, 15(S)-HETE, and arachidonic acid by 5-LO, FLAP may also function as a more general lipid carrier protein for the biosynthesis of multiple oxygenation products of arachidonic acid in addition to its role in leukotriene biosynthesis.

Leukotrienes are derived from arachidonic acid through the 5-lipoxygenase pathway. The source of arachidonic acid is obtained through hydrolysis of phospholipids by phospholipase A2 following cell stimulation (1). The free arachidonic acid is then converted by 5-lipoxygenase in the presence of 5-lipoxygenase-activating protein (FLAP)† into leukotriene A4 (LTA4) through a concerted two step reaction in which the intermediate 5-HPETE is formed (2–4). LTA4 can be further metabolized into the proinflammatory LTB4 (5) through the action of LTA4 hydrolase or into the bronchoconstrictive peptidoleukotrienes LTC4, LTD4, and LTE4, in which LTC4 synthase is the first enzyme converting LTA4 to LTC4 (6).

The 5-lipoxygenase pathway requires FLAP for efficient synthesis of leukotrienes in cellular systems (4). Recently, studies with the FLAP gene knockout mice confirm that FLAP expression is essential for leukotriene production (7). The FLAP null mice are also resistant to platelet-activating factor mediated anaphylaxis and showed reduced inflammatory responses (8). FLAP is an arachidonate binding protein that may function as an arachidonate acid transfer protein that facilitates substrate utilization by 5-LO (9, 10). An enzymatic function for FLAP has not been delineated, but the sequence similarities between LTC4 synthase and homologues of FLAP suggest that FLAP is a member of a family of microsomal glutathione S-transferases (11–14). The important role for leukotrienes in asthma has led to the development of inhibitors acting at various levels of the arachidonic acid cascade (15). Several classes of FLAP inhibitors have been developed (16–19, 20), and the mode of binding of several classes of these inhibitors to FLAP has been elucidated through site-directed mutagenesis studies (18, 19).

The leukotriene biosynthesis inhibitor MK-0591 (21) has been demonstrated to interact with FLAP. The residues 48–62 of FLAP and the negative charge of Asp62 have been shown to be essential for binding of these inhibitors (18). Various other mutants of FLAP were also constructed that did not affect the binding of inhibitors, such as MK-0591. This study focuses on the ability of various mutants of FLAP to support substrate utilization by 5-lipoxygenase when the two proteins are co-expressed using an insect cell baculovirus expression system. This co-expression system has been utilized to demonstrate that FLAP stimulates the oxygenation of exogenous arachidonic acid by 5-lipoxygenase (10). We report here that FLAP is also required for the efficient utilization of 12(S)-HETE and stimulates the use of 15(S)-HETE as a substrate for 5-lipoxygenase. Using several mutants of FLAP, this study delineates that several regions of FLAP that are not involved in inhibitor binding are essential for stimulating the oxygenation of fatty acid substrates by 5-lipoxygenase binding inhibitor.

MATERIALS AND METHODS

Chemicals and Reagents—Restriction endonucleases, DNA polymerase I, Klenow fragment, deoxy-nucleotide triphosphates, and T4 DNA ligase were obtained from Boehringer Mannheim. The radioisotopes 125I-labeled protein A (10 mCi/mg) and (α-32P)dCTP (3000 Ci/mmol) were supplied by NEN Life Science Products. Arachidonic acid, 12(S)-HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; del, deletion.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, P. O. Box 1005, Pt. Claire/Dorval, Quebec H9R 4P8, Canada.

† The abbreviations used are: LT, leukotriene; FLAP, 5-lipoxygenase-activating protein; 5-LO, 5-lipoxygenase; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; del, deletion.
HETE, 15(S)-HETE, 5(S),12(S)-dihETE, and 5(S),15(S)-dihETE were obtained from Cayman Chemical Co. (Ann Arbor, MI).

Cells and Viruses—Sf9 insect cells and the pETL-FLAP transfer vector were kindly provided by Dr. C. D. Richardson (Biotechnology Research Institute, Montreal, Quebec, Canada). Sf9 insect cells were cultured in Grace’s complete medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin sulfate, and 2.5 μg/ml amphotericin B (Fungizone) in either Corning T flasks (Richmond Hill, Ontario) or Corning spinner flasks at 27 °C following the procedures of Summers and Smith (22). Cell viability (>96%) was determined using 0.2% Trypan blue using a hemocytometer.

Construction of Recombinant Transfer Vector pETL-FLAP—The human FLAP cDNA mutants were subcloned from the vector pBSShFLAP (plasmid Bluscript human FLAP) into the BanHI site of pETL DNA preparations of the bacterial transformants were prepared, and the proper orientation in relation to the polyhedrin promoter was determined by restriction endonuclease mapping. The recombinant transfer vector (2 μg) was mixed with linearized wild type Autographa californica nuclear polyhedrosis virus (AcMNPV) DNA (1 μg) (Invitrogen, San Diego, CA) and co-transfected into Sf9 cells by the calcium phosphate method according to the manufacturer’s instructions. The recombinant baculovirus was obtained from the supernatant by centrifugation of the Sf9 cells and media at 1000 × g for 5 min. Plaque assays were performed in culture dishes as described previously (10). Ten plaques were isolated from the first plaque assay and subjected to dot blot analysis using a radiolabeled human FLAP (hFLAP) cDNA fragment. A second round of plaque purification was performed and the isolated virions were titered and utilized to co-express with 5-LO. Recombinant baculovirus preparations were used at a multiplicity of infection of 5 for infection of Sf9 cells. Recombinant viral 5-LO and wild type FLAP were previously described (10).

Eicosanoid Synthesis—Sf9 cells were harvested by centrifugation (300 × g for 5 min at room temperature) and washed with Dulbecco’s phosphate-buffered saline (Life Technologies, Inc.). Small aliquots of these cells were retained for immunoblot analysis. The remaining cells were resuspended at a cell density of 1–10 × 10⁶ cells/ml in Dulbecco’s phosphate-buffered saline, prewarmed to 37 °C. One-ml aliquots were challenged with 10 μM A23187 and either arachidonic acid, 12(S)-HETE, or 15(S)-HETE. Following incubation at 37 °C for 10 min, the reaction was terminated by addition of 1 ml of methanol containing 0.25 nmol of prostaglandin B₃ (internal standard for HPLC analysis). Eicosanoids were extracted with chloroform, dried under nitrogen, and reuspended in HPLC solvent (methanol/water/acetic acid, 75/25/0.01). The products were analyzed by reverse phase HPLC using a Nova-Pak C₁₈ column (Waters, Mississauga, Ontario, Canada) and an isocratic HPLC solvent system (see above) at a constant flow rate of 1 ml/min. The eluant was monitored for conjugated trienes and dienes at wavelengths of 270 and 234 nm, respectively, using a model 991 photodiode array system (Waters). Products were quantitated by integration of peak areas as compared with authentic standards.

Protein Immunoblotting—Sf9 cells expressing the various recombinant proteins were harvested by centrifugation (300 × g for 5 min at room temperature) and washed once with Dulbecco’s phosphate-buffered saline (Life Technologies, Inc.). Cells were then sonicated in 15 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride at 4 °C and centrifuged at 100,000 × g for 60 min. Protein concentrations were determined in microtitre plates using Coomassie protein assay reagent (Pierce) according to the manufacturer’s instructions. The supernatant and pellet fractions were analyzed for expression of 5-LO and FLAP, respectively. Protein samples were separated on 14% polyacrylamide gels (Novex, San Diego, CA) according to the manufacturer’s instructions. Samples were electropheretically transferred to nitrocellulose overnight. Immunoblot analysis was performed as described previously (23) using a 1:200 dilution of antiserum and ¹²⁵I-labeled protein A.

RESULTS

Utilization of Arachidonic Acid by 5-LO in the Presence of FLAP or FLAP Mutants—Cellular leukotriene synthesis requires both 5-LO and its activating protein (FLAP). Previous work on the co-expression of 5-LO and FLAP in Sf9 insect cells has demonstrated that FLAP stimulates the use of arachidonic acid as a substrate and increases the efficiency with which 5-LO converts 5-H(π)ETE to LTA₄ (10). In the present study, we have co-expressed 5-LO with different FLAP mutants in order to determine their ability to support eicosanoid formation when co-expressed with 5-LO. The levels of FLAP mutants and 5-LO expression were evaluated by immunoblot analysis (Fig. 1) in the same Sf9 cells that were utilized for the measurement of eicosanoid production. The amount of FLAP expression in the insect cell preparations was very similar for the various mutants (hFLAP (del 37–53), hFLAP (del 52–58), hFLAP D62N, hFLAP (del 106–108), and hFLAP (del 148–161)) (Fig. 1B). The level of 5-LO expression varied between the different infections and was generally lower when the enzyme was co-expressed with FLAP rather than alone (Fig. 1A). However, the cells containing the lowest amount of 5-LO (Fig. 1, 5-LO + hFLAP D62N) synthesized 5-H(π)ETE and LTA₄ to amounts comparable to when 5-LO was co-expressed with wild type FLAP, suggesting that FLAP rather than 5-LO levels are limiting for product formation.

The Sf9 cells expressing 5-LO and the various FLAP mutants were challenged with a mixture of 10 μM arachidonic acid and 10 μM A23187, and the reaction products were analyzed by reverse phase HPLC (Fig. 2). Arachidonic acid was required because no detectable product formation was detected when cells were challenged only with the calcium ionophore A23187 (data not shown), as previously reported (10). Two of the major products detected were 5-H(π)ETE (Fig. 2, peak 6), and LTA₄ (measured as the nonenzymatic hydrolysis products 6-trans-LTB₄ and 6-trans-12-epi-LTB₄; Fig. 2, peaks 2 and 3). In addition, two products that comigrated with authentic 5,6-diHETE were detected (Fig. 2, peaks 4 and 5). LTB₄ synthesis was not observed in these cells because LTA₄ hydrolase is not present in Sf9 insect cells (24).

Fig. 2 also shows that the cells co-expressing 5-LO and FLAP produced higher levels of both 5-H(π)ETE and LTA₄ than cells expressing only 5-LO when challenged with arachidonic acid and calcium ionophore. The data for the effect of FLAP and of the various FLAP mutants on the production of 5-H(π)ETE and LTA₄ by Sf9 are summarized in Table I. The co-expression of FLAP with 5-LO resulted in 3-fold and 9-fold increases in the
production of 5-H(P)ETE and LTA₄, respectively. The ratio of LTA₄ to 5-H(P)ETE increased to 0.7 in the co-expressing cells as compared with a ratio of 0.25 in cells expressing 5-LO alone. In contrast to FLAP, the mutant hFLAP (del 37–53) co-expressed with 5-LO in SF9 cells resulted in a stimulation of product formation of approximately 3-fold above 5-LO alone and had little effect on the ratio of LTA₄ to 5-H(P)ETE (ratio of 0.28 versus 0.25 for SF9 cells expressing only 5-LO). The hFLAP (del 106–108) and (del 148–161) caused less than a 2-fold stimulation of either 5-H(P)ETE or LTA₄ synthesis, and the ratios of LTA₄ to 5-H(P)ETE were 0.26 and 0.37 for the two deletions, respectively. These results suggest that the residues 106–108 and 148–161 of FLAP are important for efficient transfer of arachidonic acid from FLAP to 5-LO.

Co-expression of 5-LO with hFLAP (del 52–58) in SF9 cells challenged as above results in an increased production of 5-H(P)ETE (2-fold) and LTA₄ (9-fold) with an increased ratio of LTA₄ to 5-H(P)ETE of 1.25. An interesting point mutant in FLAP demonstrated complete loss of inhibitor binding when the negative charge of the residue was neutralized (18). This mutant hFLAP D62N was co-expressed with 5-LO in SF9 cells and showed no difference in arachidonic acid utilization by 5-LO as compared with the wild type FLAP (Table I). These data indicate that amino acids 52–58 and 62 are not essential for an efficient transfer of arachidonic acid to 5-LO by FLAP.

**FLAP-dependent Oxygenation of 12(S)-HETE by 5-Lipoxigenase**—It has previously been shown that purified 5-LO can utilize 12(S)- and 15(S)-HETE as substrates in phospholipid vesicles (25). Using the SF9 insect cell expression system, we asked the question of whether FLAP was essential for the cellular oxygenation of 12(S)-HETE by 5-lipoxigenase. When 5-LO was expressed in SF9 insect cells in the absence of FLAP and challenged with 10 μM 12(S)-HETE and A23187, no product formation could be detected by reverse phase HPLC (Fig. 3, left panel). In contrast, a significant amount of 5,12-diHETE was synthesized in a similar experiment in which both 5-LO and FLAP were co-expressed. The synthesis of 5,12-diHETE increased with the 12(S)-HETE concentration for the cells co-expressing 5-LO and FLAP, with significant product formation reaching a plateau at a substrate concentration of 15–30 μM, whereas no significant product synthesis was detected for cells expressing only 5-LO over the range of 1–60 μM 12(S)-HETE (Fig. 3, right panel). Also, when the FLAP inhibitor MK-886 (10 μM) was preincubated with cells co-expressing 5-LO and FLAP prior to challenge with 12(S)-HETE, 5,12-diHETE formation was completely inhibited (Fig. 3, right panel). The MK-886-inhibitable diHETE formation is consistent with a FLAP-dependent 12(S)-HETE oxygenation by 5-lipoxigenase. SF9 cells expressing FLAP alone were tested for the ability to utilize 12(S)-HETE as a substrate, but no product formation could be detected (data not shown).

Oxygenation of 12(S)-HETE by SF9 Cells Co-expressing 5-LO and FLAP Mutants—The five FLAP mutants were each co-expressed with 5-LO in SF9 insect cells and challenged with 10 μM 12(S)-HETE and 10 μM A23187. Representative HPLC tracings of product formation for three of the co-infection experiments are presented in Fig. 4. These results show that 5-LO co-expressed with FLAP del 37–53 produces no significant conversion of 12(S)-HETE into 5,12-diHETE. In contrast, 5-LO co-expressed with FLAP D62N results in complete conversion of 12(S)-HETE into 5,12-diHETE. The hFLAP (del 52–58) showed partial activity and resembled to or slightly higher than that seen for the wild type FLAP. The results for the various FLAP mutants are presented in Table II. The SF9 cells infected with 5-LO alone converted an insignificant amount of the substrate 12(S)-HETE into the product 5,12-diHETE. Expression of any of the three mutants, hFLAP (del 37–53), hFLAP (del 106–108), or hFLAP (del 148–161) with 5-LO results in no significant increase in utilization of the 12(S)-HETE substrate. Co-expression of 5-LO with the single point mutant hFLAP D62N results in product formation equivalent to or slightly higher than that seen for the wild type FLAP. The hFLAP (del 52–58) showed partial activity and stimulated 5,12-diHETE formation to about 15% of the level obtained for cells expressing 5-LO and wild type FLAP.

Two other deletions located, respectively, between the second and third transmembrane domains (del 106–108) and at the C-terminal tail (del 148–161) have been previously reported to have no effect on inhibitor binding (18). When these mutants were each separately co-expressed with 5-LO in SF9 cells and challenged with either arachidonic acid or 12(S)-HETE and A23187, no stimulation of substrate utilization was observed as compared with SF9 cells expressing only 5-LO. This demonstrates that these mutations affect the ability of FLAP to increase the utilization of substrates by 5-LO and suggest an-
FLAP-dependent Oxygenation of 12-HETE and 15-HETE by 5-LO

The production of 5-H(PE)TE and LTA₄ by SF9 cells co-expressing 5-LO and FLAP mutants and stimulated with calcium ionophore and arachidonic acid was measured by reverse phase HPLC as shown in Fig. 2. Each value represents the average of two determinations.

| Cellular expression | 5-H(P)ETE nmol/million cells | LTA₄ nmol/million cells | LTA₄/5-H(P)ETE ratio |
|---------------------|-----------------------------|------------------------|---------------------|
| 5-LO                | 0.015                       | 0.0037                 | 0.25                |
| 5-LO and hFLAP      | 0.051                       | 0.034                  | 0.7                 |
| 5-LO and hFLAP (del 37–53) | 0.037                  | 0.01                   | 0.28                |
| 5-LO and hFLAP (del 52–58) | 0.025               | 0.034                  | 1.2                 |
| 5-LO and hFLAP D62N | 0.052                       | 0.041                  | 0.77                |
| 5-LO and hFLAP (del 106–108) | 0.022              | 0.0057                 | 0.26                |
| 5-LO and hFLAP (del 148–161) | 0.0144              | 0.0054                 | 0.37                |

**FIG. 3. Synthesis of 5,12-diHETE by 5-lipoxygenase.** SF9 cells expressing 5-LO or 5-LO and FLAP were challenged with 10 μM A23187 and 10 μM 12(S)-HETE (+/+ MK-886). The product formation was monitored by UV absorbance after separation by reverse phase HPLC (left panel). The designated peaks correspond to PGB₂, internal standard (peak 1) and 5(S)12(S)-diHETE (peak 2). The graph on the right represents the product accumulation at various concentrations of substrate (12(S)-HETE). Product formation by SF9 cells co-expressing 5-LO and FLAP ( peaks) are depicted. SF9 cells expressing 5-LO alone (○) or 5-LO and FLAP in the presence of MK-886 (●) did not produce significant amounts of 5,12-diHETE (overlapping symbols).

**TABLE I**

Effect of FLAP mutants on arachidonic acid oxidation by 5-LO

15(S)-HETE Utilization by SF9 Cells Expressing 5-LO and FLAP—15(S)-HETE is a precursor to lipoxin formation and the corresponding 5,15-diHETE has been implicated in inflammatory disorders such as rheumatoid arthritis (26). We investigated the utilization of 15(S)-HETE as a substrate in our cellular expression system of 5-LO and FLAP. When SF9 cells expressing only 5-LO were challenged with 10 μM A23187 and various concentrations of 15(S)-HETE, a significant amount of 5,15-diHETE was formed, with maximal product formation occurring at a substrate concentration between 7–10 μM (Fig. 5). When cells co-expressing 5-LO and FLAP were challenged in a similar fashion, a 2–2.5-fold increase of product formation was detected. This increase in 5,15-diHETE formation was inhibitable by MK-886 to levels detected in cells expressing 5-LO alone. This shows that FLAP stimulates cellular 15(S)-HETE utilization by 5-LO, but it is not essential for oxygenation to 5,15-diHETE.

**DISCUSSION**

5-Lipoxygenase-activating protein is a membrane protein required for leukotriene synthesis, and its functional role may be an arachidonate/lipid transfer protein (9). This study demonstrates that FLAP allows 5-lipoxygenase to utilize the substrate 12(S)-HETE for formation of 5,12-diHETE and stimulates the oxygenation of 15(S)-HETE by 5-LO. This also is the first delineation of residues of FLAP involved in eicosanoid synthesis, utilizing either 12(S)-HETE or arachidonic acid as a substrate. The residues of FLAP important for eicosanoid formation are compared with the residues essential for inhibitor binding. Based upon the mutants utilized in this study, the proposed inhibitor interaction sites overlap with the arachidonate interaction sites, but some unique residues are responsible for inhibitor binding as compared with arachidonate binding.

It has been reported previously that the membrane association of 5-lipoxygenase from the cytosol fraction changes the substrate specificity of 5-lipoxygenase and allowed the enzyme to utilize 12(S)-HETE and 15(S)-HETE as substrates (27). Utilizing the SF9 cells, activation of 5-LO following calcium ionophore treatment resulted in no significant 12(S)-HETE substrate utilization. This substrate was efficiently utilized only in cells co-expressing both 5-LO and FLAP. The requirement of FLAP for utilization of 12(S)-HETE and stimulation of 15(S)-HETE as substrates for 5-LO in SF9 cells suggests that FLAP, which appears to act as an arachidonate transfer protein, may transfer other lipid molecules to 5-LO for product formation.

The ability of FLAP to stimulate the utilization of oxygenated eicosanoids may have important implications with regard to the mechanism by which FLAP increases the ratio of LTA₄/5-H(PE)TE production. 5-LO in cell free systems produced variable ratios of 5-H(PE)TE to LTA₄ depending on assay conditions (28), with some 5-H(PE)TE being channeled to LTA₄ by 5-LO. Arachidonic acid and 5-H(PE)TE can compete for utilization by 5-LO (29), and a high ratio of accumulation of LTA₄ to 5-H(PE)TE can be observed under conditions of limiting...
arachidonic acid availability. Binding of the 5-H(P)ETE intermediate and transfer to 5-LO by FLAP before its reduction to 5-HETE could provide a mechanism to increase the LTA4 to 5-H(P)ETE ratio observed in cell systems.

Assays with the 12(S)-HETE substrate and arachidonic acid were used in an Sf9 co-expression system to determine which residues of FLAP were essential for 5,12-diHETE product formation and efficient LTA4 production from arachidonic acid. The results of these experiments are summarized in Table III. The FLAP mutants D62N and del 52–58 were similar to the wild type FLAP in stimulating the use of 12(S)-HETE as a substrate by 5-LO and increasing the efficiency of leukotriene production by 5-LO with exogenous arachidonic acid. This is in contrast to residues involved in inhibitor binding because these FLAP mutants have lost the ability to bind indole inhibitors (18, 19). The remaining FLAP mutants, del 37–53, del 106–108, and del 148–161, are all defective in supporting substrate utilization by 5-LO, although these residues could be essential for the overall structural integrity of FLAP. It is worthwhile to note that del 106–108 and del 148–161 do not effect the binding of analogues of MK-0591 to FLAP (19), suggesting that these deletions do not alter the conformation of FLAP as determined by inhibitor binding (see Table III). In addition, the mutant del 37–53 was found to be deficient in inhibitor binding (18), demonstrating a direct overlap of residues involved in inhibitor binding and eicosanoid formation and in agreement with previous data on competition between MK-886 and the photoaffinity analogue of arachidonic acid (9). The mutants del 106–108 and del 148–161 are not deficient in inhibitor binding, but another mutation in this vicinity, Y101A, abolishes all inhibitor binding.2 The putative transmembrane model of FLAP suggests that residues 94–117 are on the opposite membrane bilayer with respect to residues 29–62 and 148–161, which are on the same membrane bilayer (18). We therefore propose that the second putative transmembrane domain may span as a loop through the membrane and place residues 106–108 on the same side as the C-terminal tail (Fig. 6). This conformation results in all the residues of FLAP that are essential for either eicosanoid utilization by 5-LO or inhibitor binding to exist on the same side of the membrane bilayer.

Table II

| Cellular expression | 5,12-diHETE nmol/million cells |
|---------------------|-------------------------------|
| 5-LO                | 0.001                         |
| 5-LO and hFLAP      | 0.17                          |
| 5-LO and hFLAP (del 37–53) | 0.002                   |
| 5-LO and hFLAP (del 52–58) | 0.02                   |
| 5-LO and hFLAP D62N | 0.22                          |
| 5-LO and hFLAP (del 106–108) | 0.002                  |
| 5-LO and hFLAP (del 148–161) | 0.001                  |

residues of FLAP were essential for 5,12-diHETE product formation and efficient LTA4 production from arachidonic acid. The results of these experiments are summarized in Table III. The FLAP mutants D62N and del 52–58 were similar to the wild type FLAP in stimulating the use of 12(S)-HETE as a substrate by 5-LO and increasing the efficiency of leukotriene production by 5-LO with exogenous arachidonic acid. This is in contrast to residues involved in inhibitor binding because these FLAP mutants have lost the ability to bind indole inhibitors (18, 19). The remaining FLAP mutants, del 37–53, del 106–108, and del 148–161, are all defective in supporting substrate utilization by 5-LO, although these residues could be essential for the overall structural integrity of FLAP. It is worthwhile to note that del 106–108 and del 148–161 do not effect the binding of analogues of MK-0591 to FLAP (19), suggesting that these deletions do not alter the conformation of FLAP as determined by inhibitor binding (see Table III). In addition, the mutant del 37–53 was found to be deficient in inhibitor binding (18), demonstrating a direct overlap of residues involved in inhibitor binding and eicosanoid formation and in agreement with previous data on competition between MK-886 and the photoaffinity analogue of arachidonic acid (9). The mutants del 106–108 and del 148–161 are not deficient in inhibitor binding, but another mutation in this vicinity, Y101A, abolishes all inhibitor binding.2 The putative transmembrane model of FLAP suggests that residues 94–117 are on the opposite membrane bilayer with respect to residues 29–62 and 148–161, which are on the same membrane bilayer (18). We therefore propose that the second putative transmembrane domain may span as a loop through the membrane and place residues 106–108 on the same side as the C-terminal tail (Fig. 6). This conformation results in all the residues of FLAP that are essential for either eicosanoid utilization by 5-LO or inhibitor binding to exist on the same side of the membrane bilayer.

Leukotriene C4 synthase contains significant sequence homol-

2 J. A. Mancini and S. Charleson, unpublished results.
This model is analogous to the recently proposed membrane transducer as the hydrophilic stretch after the first transmembrane domain 2 and 3 of FLAP is located on the same side of the membrane as the hydrophilic loop between transmembrane domains 2 and 3. The diagram demonstrates that the hydrophilic loop between transmembrane domains 2 and 3 of FLAP is located on the same side of the membrane as the hydrophilic stretch after the first transmembrane domain. This model is analogous to the recently proposed membrane topology of LTC4 synthase (30).

With FLAP, a recent study of site-directed mutagenesis of LTC4 synthase has led to the proposal that the first two hydrophilic loops (corresponding to the equivalent loops in FLAP) are important for catalytic function of the enzyme and that they exist on the same side of the membrane (30), similar to the present proposed topology of FLAP.

This study demonstrates that FLAP can stimulate both 12(S)-HETE and 15(S)-HETE utilization by 5-LO in arachidonic acid, and suggests that FLAP acts as a transfer protein for lipids other than arachidonic acid. The utilization of 12(S)-HETE as a substrate by 5-LO shows a greater dependence on FLAP than 15(S)-HETE. This is consistent with data in the literature, which show that in a purified enzyme assay 5-LO can utilize 15(S)-HETE as a substrate with an efficiency that is approximately 30% as efficient as a substrate for 5-LO as arachidonic acid (25). The ability of FLAP to play a role in the dioxygenation of eicosanoid substrate suggests that FLAP may also be important for 5,15-diHETE and lipoxin formation. An increase synthesis of 5,15-diHETE has been implicated in rheumatoid arthritis (26), and this correlates with the FLAP-deficient mice, which have a decrease response in collagen-induced arthritis (a model for rheumatoid arthritis) (8). Using

| Cellular expression | 5,12-diHETE | LTA4/HETE ratio | Inhibitor binding |
|---------------------|-------------|-----------------|------------------|
| 5-LO                | +           | +               | +                |
| 5-LO and hFLAP      | ++          | +++             | +++              |
| 5-LO and hFLAP (del 52–58) | +     | +               | +                |
| 5-LO and hFLAP D62N | +           | +               | +                |
| 5-LO and hFLAP (del 106–108) | +   | +               | +                |
| 5-LO and hFLAP (del 148–161) | + | +           | +                |
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Joseph A. Mancini, Heather Waterman and Denis Riendeau

J. Biol. Chem. 1998, 273:32842-32847.
doi: 10.1074/jbc.273.49.32842

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