Characterization of the Lysyl Adducts of Prostaglandin H-Synthases That Are Derived from Oxygenation of Arachidonic Acid†

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Received November 15, 2000; Revised Manuscript Received April 18, 2001

ABSTRACT: These investigations characterize the covalent binding of reactive products of prostaglandin H-synthases (PGHSs) to the enzyme and to other molecules. The intermediate product of oxygenation of arachidonic acid by the PGHSs, prostaglandin (PG) H_2, undergoes rearrangement to the highly reactive γ-keto aldehydes, levuglandin (LG) E_2 and D_2. We previously have demonstrated that LGE_2 reacts with the ϵ-amine of lysine to form both the lysyl−levuglandin Shiff base and the pyrrole-derived lysyl−levuglandin lactam adducts. We now demonstrate that these lysyl−levuglandin adducts are formed on the PGHSs following the oxygenation of arachidonic acid; after reduction of the putative Schiff base, proteolytic digestion of the enzyme, and isolation of the adducted amino acid residues, these adducts were identified by liquid chromatography−tandem mass spectrometry. The reactivity of the LGs is reflected by the finding that virtually all of the LG predicted to be formed from PGH_2 can be accounted for as adducts of the PGH-synthase and that oxygenation of arachidonic acid by PGH-synthases also leads to the formation of adducts of other proteins present in the reaction solution. The reactivity of the PGH-synthase adducts themselves is demonstrated by the formation of intermolecular cross-links.

The endoperoxide prostaglandin H_2 is the product of oxygenation of arachidonic acid by the two isoenzymes, prostaglandin H-synthase (PGHS)[1] 1 and 2. Prostaglandin (PG) H_2 is further metabolized to PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxane A_2, and prostacyclin by specific synthases or reductase. PGH_2 in aqueous solutions rearranges to PGE_2 and PGD_2, and 20% of it is converted into the highly reactive γ-keto aldehydes, levuglandin (LG) E_2 and D_2 (*1*, *2*) (Figure 1). Oxygenation of arachidonic acid by PGHS-1 has been shown to lead to formation of a reactive product(s) that adducts both to PGHS-1 itself and to other proteins in solution with the enzyme (*3*). Further, oxygenation of arachidonic acid by PGHS-1 in platelet's microsomes produces arachidonic acid-derived adducts of multiple proteins (*4*). Such labeling also has been reported using whole platelets (*5*), even though thromboxane A_2 synthase is coexpressed. However, the reactive product of arachidonic acid that forms these protein adducts has not yet been characterized.

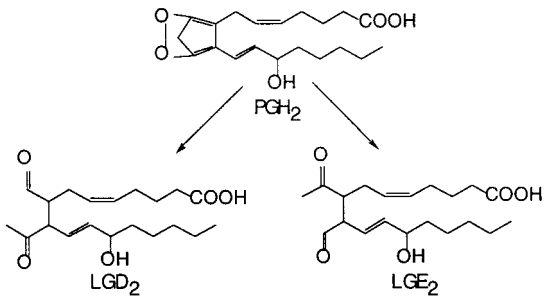

FIGURE 1: Structure of levuglandins E_2 and D_2.

We hypothesized that these adducts are formed from LGE_2 and LGD_2. The levuglandins are highly reactive, and LGE_2 is known to react covalently with the ϵ-amine of lysine, with proteins, and with DNA (*6*, *7*). As a basis for determining whether the arachidonic acid-derived adducts of proteins are formed from levuglandins, we previously have characterized the adducts of lysine that are formed by the reaction of that amino acid with LGE_2 or PGH_2 (*2*, *8*) (Figure 2). The lysyl−LG Schiff base is the immediate product of this reaction, whereas the pyrrole-derived lactam and hydroxylactam adducts are formed subsequently as levels of the Schiff base decline.

This report provides evidence indicating the presence of two lysyl−LG adducts on PGHS-1 and PGHS-2 that are

† This work was supported in part by NIH Grants CA 68485, GM 15431, CA47479, and GM 58008-01. J.A.O. is the Thomas F. Frist Sr. Chair in Medicine.

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[1] Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DMF, dimethylformamide; GC, gas chromatography; LC−ESI-MS/MS, liquid chromatography−electrospray ionization tandem mass spectrometry; LG, levuglandin; MALDI, matrix-assisted laser desorption ionization; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MS, mass spectrometry; PBS, phosphate-buffered saline; PE, polyethylene; PG, prostaglandin; PGHS, prostaglandin H-synthase; RP-HPLC, reversed phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

10.1021/bi002629k CCC: $20.00
Published on Web 05/17/2001

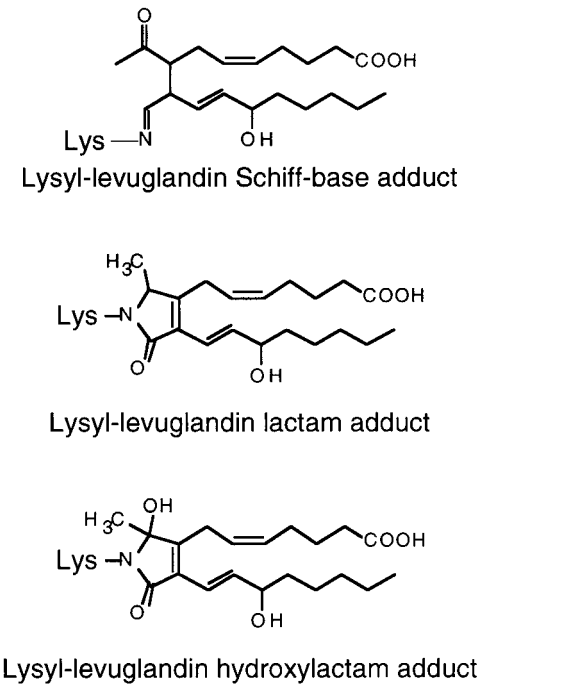

FIGURE 2: Structures of the lysyl−LG adducts that have been characterized.

formed as a consequence of the oxygenation of arachidonic acid. Other proteins that are in solution with a PGHS during catalysis are adducted as well. The participation of these adducts in intermolecular cross-linking also has been addressed.

EXPERIMENTAL PROCEDURES

Materials. PGH_2 was from Cayman Chemical Co. (Ann Arbor, MI). Methoxylamine hydrochloride, diisopropylethylamine, pentaflurobenzyl bromide, undecane, lysine, sodium cyanide, sodium borohydride, sinapinic acid, bovine serum albumin, [^{14}C]ubiquitin, and sodium borodeuteride were purchased from Sigma (St. Louis, MO). Oasis Sep Pak cartridges were obtained from Waters Corp. (Milford, MA), and dimethyl formamide was from Aldrich Chemical Co. (Milwaukee, WI). Bis(trimethylsilyl)trifluoroacetamide was from Supelco (Bellefonte, PA). Pronase and aminopeptidase M from porcine kidney were from Calbiochem (San Diego, CA). [^{14}C]Spermine tetrahydrochloride was from Amersham (Piscataway, NJ), and [^{14}C]arachidonic acid was from NEN (Boston, MA). PGHS-1 was purified from ram seminal vesicles as previously described (*9*). Wild-type mouse PGHS-2 was expressed in SF-9 cells (Novagen, Madison, WI) and purified as previously described (*10*).

Incubation of PGHS-2 with [^{14}C]Arachidonic Acid. Wild-type mouse PGHS-2 (14 μM) was preincubated at 37 °C for 5 min in 100 mM oxygenated phosphate buffer (pH 7.5) and 500 μM phenol. At this time, 2 molar equiv of hematin was added, and the mixture was incubated at 37 °C for an additional 1 min, then transferred to a tube containing 4.8 μCi of [^{14}C] arachidonic acid (944 μM, final concentration), and incubated at 37 °C for the desired amount of time.

Chromatography. After incubation, the enzyme was or was not dialyzed at 4 °C overnight against 100 mM sodium phosphate buffer (pH 8.0), 50 mM NaCl, 0.4% CHAPS, and 500 μM phenol. An aliquot was then applied to a 4.6 mm × 250 mm Protein C4 VYDAC column (VYDAC, Hesperia, CA) with a flow rate of 1.0 mL/min, and the adducts were eluted with a linear gradient of 50 to 75% acetonitrile in a water/0.1% TFA mixture over the course of 30 min. The amount of radioactivity was monitored using a radioactive flow detector Flo-One βeta Model CT (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL).

Mass Spectrometry. A carbon-embedded polyethylene (PE) membrane was used as a sample support (*11*, *12*) for matrix-assisted laser desorption ionization (MALDI) mass spectrometry analysis (MS) (*13*, *14*). Conductive PE was purchased in thin sheet format (80 μm) from Goodfellow Cambridge Ltd. (Cambridge, England). The membrane was mounted on the target holder using double-side conductive tape (3M Co.). The hydrophobic and electrostatic interactions between the sample molecule and the PE membrane allow for sample washing, which removes any excess of salts and buffers incompatible with the MALDI process when present in large amounts. The samples were deposited on the membrane and allowed to dry at room temperature. The blotted areas were then rinsed with 20 μL of water using an automatic pipet. Water was deposited and removed from the membrane several times over a period of 30 s. When the samples had been dried from rinsing, 1 μL of the matrix (sinapinic acid at a concentration of 10 mg/mL in a 1/1 mixture of acetonitrile and 0.1% TFA in H_2O) was deposited on the blotted areas and allowed to dry prior to mass spectrometry.

The samples were analyzed using a Perseptive DE-STR MALDI time-of-flight mass spectrometer (Applied Biosystem, Framinglam, MA). The instrument was operated in the linear mode at 25 kV under optimized delayed extraction conditions. The mass spectra have been obtained by averaging 256 individual laser shots and were further processed using Data Explorer (Applied Biosystem). Mass calibration was achieved using the doubly charged and molecular ions of bovine serum albumin (BSA, MW of 66 430). BSA was prepared in 0.1% TFA in H_2O at a final concentration of 5 pmol/μL. One microliter of this solution was codeposited with sinapinic acid as the matrix on the sample target plate used for sample analysis. The centroid peak values as well as the standard deviations have been determined by averaging five independent measurements.

Kinetics of Formation of the Adducts on PGHS-2. PGHS-2 (14 μM) was preincubated as described above. Then 0, 10, 30, 40, and 60 s and 2, 5, and 10 min after addition of the arachidonic acid, 10 μL of the reaction mixture was taken and mixed with 10 μL of 100 mM sodium borohydride in 0.1 N sodium hydroxide. The reduction of the prostanoids was allowed to occur at 4 °C for 30 min, and the samples were then frozen until analysis by chromatography on a 4.6 mm × 250 mm Protein C4 VYDAC column. The solvent system consisted of a linear gradient of 0 to 75% acetonitrile in 0.1% TFA in water over the course of 30 min. The level of radioactivity and the absorbance at 230 nm were monitored. The amount of protein was determined using the area of the UV peak, and the oxygenation products were assessed by using the radioactive signals.

Calculation of the Stoichiometry of the Adducts on PGHS-2. After the reaction, the mixture was injected onto a reversed phase C4 HPLC system and the amount of radioactivity monitored in line. For each time point, the amount of protein

was calculated from the area of the UV peak at 230 nm in relation to that of unreacted PGHS-2, the radioactive peaks were integrated, and the values that were obtained were used for the calculation of the stoichiometry. Under these conditions, 59 nmol of arachidonic acid was oxygenated per nanomole of PGHS-2 (8.45 nmol of products for 143 pmol of enzyme).

Characterization of Arachidonic Acid-Derived Adducts of PGHS-1 and PGHS-2. Wild-type mouse PGHS-2 (14.2 μM) or ovine PGHS-1 (14.2 μM) was preincubated for 5 min at 37 °C in 100 mM oxygenated phosphate buffer (pH 7.5) containing 500 μM phenol and 25 μM hematin (2 equiv of PGHS-2). Then, arachidonic acid (944 μM, final concentration) was added, and the mixture was incubated at 37 °C for 20 min. The [^{13}C,^{3}H]lysyl−LG lactam adduct (14.5 ng, 22 200 cpm) and the [^{13}C,^{3}H]lysyl−LG reduced Schiff base adduct (20 ng, 33 200 cpm) were added as internal standards, and the reaction was stopped by adding sodium borohydride in DMF at a final concentration of 20 mM. After 30 min at room temperature, the pH of the solution was neutralized with 20 μL of 10× PBS, and PGHS-2 was denatured at 95 °C for 5 min. After a first digestion by Pronase (1/5 enzyme/substrate ratio) under argon at 37 °C overnight, followed by denaturation at 95 °C for 5 min, the peptides were further digested to single amino acids under argon by incubation for 24 h at 37 °C with aminopeptidase M from porcine kidney (0.1 unit). The peptidase was then inactivated at 95 °C for 5 min.

The lysyl adducts were purified by solid phase extraction using the Oasis SepPak column. After being washed with water, the adducts were eluted with methanol. The volume of the solvent was decreased to ~100 μL and diluted with 1.5 mL of water.

The two adducts were then separated by C18 RP-HPLC on a macrosphere 300 C18 5 μm column (4.6 mm × 250 mm) from Alltech Associates, Inc. (Deerfield, IL). The conditions of chromatography are as follows. The column was washed at a rate of 1 mL/min for 10 min with a 20 mM ammonium acetate/acetic acid mixture (100/0.1). Then, the mobile phase was exchanged in 10 min with a linear gradient to a methanol/20 mM ammonium acetate/acetic acid mixture (50/50/0.1), and was kept as such for an additional 50 min. Fractions of 2 mL were collected, and 50 μL of each was counted with a scintillation counter. The radioactive fractions were pooled for the two peaks and concentrated by solid extraction using an Oasis SepPak column as previously described. After concentration of the eluate under a nitrogen stream, the samples were analyzed by liquid chromatography−electrospray ionization-tandem mass spectrometry (LC−ESI-MS/MS).

LC−MS/MS Analysis. The adducts of lysine derived from protease digestion of the cyclooxygenases were chromatographed on a 2.1 mm × 15 mm XDB C8 column (MacMod Analytical) with a flow rate of 0.2 mL/min using a linear gradient of 10 to 90% acetonitrile in a 5 mM ammonium acetate/0.1% acetic acid mixture. Electrospray tandem mass spectrometric analysis was carried out on a Finnigan TSQ7000 system (San Jose, CA). The sheath gas pressure was held at 70 psi; the auxiliary gas pressure at 10 psi. The spray voltage was held at 3.8 eV. The voltage on the capillary was 20 V and on the tube lens 75 V. Ions were subjected to collision-induced dissociation at −28 eV (*m*/*z* 467.4, reduced Schiff base adduct) or −36 eV (*m*/*z* 479.4, lactam adduct) with 3.2 mTorr collision gas, monitoring the daughters at *m*/*z* 321.4 and 84.1, respectively.

Formation of Arachidonic Acid-Derived Adducts of Histones. PGHS-2 was incubated with [^{14}C]arachidonic acid in the presence of a mixture of histone fractions IIA and IIIS (Sigma) from calf thymus as described above. After incubation for 30 min, the reaction mix was denatured at 70 °C in the presence of NuPAGE LDS-loading buffer and loaded onto a 10% acrylamide NuPAGE Bis-Tris gel from Novex (San Diego, CA). The proteins were separated by electrophoresis using the NuPAGE MOPS running buffer from Novex. At the end of the electrophoresis, the proteins were stained with Coomassie Blue. The amount of radioactivity associated with the proteins was determined by autoradiography.

Formation of Arachidonic Acid-Derived Adducts of Ubiquitin. PGHS-2 was incubated with [^{14}C]arachidonic acid in the presence of ubiquitin as described above. After incubation for 30 min, the reaction mix was denatured at 70 °C in the presence of LDS-loading buffer and loaded onto a 4 to 12% acrylamide NuPAGE Bis-Tris gel. The proteins were separated by electrophoresis using the NuPAGE MOPS running buffer. At the end of the electrophoresis, the proteins were stained with Coomassie Blue. The amount of radioactivity associated with the proteins was determined by autoradiography.

Participation of PGHS-2-Derived Arachidonic Acid Products in Covalent Binding of [^{14}C]Spermine to Albumin. PGHS-2 was incubated with arachidonic acid and BSA (10 μM) in the presence of [^{14}C]spermine as described above. After incubation for 30 min, the reaction mix was denatured at 70 °C in the presence of LDS-loading buffer and loaded onto a 4 to 12% acrylamide NuPAGE Bis-Tris gel. The proteins were separated by electrophoresis using the NuPAGE MOPS running buffer. At the end of the electrophoresis, the proteins were stained with Coomassie Blue. The amount of radioactivity associated with the proteins was determined by autoradiography.

Participation of LGE_2 in Covalent Binding of [^{14}C]-Spermine to Albumin. BSA (10 μM in PBS) was incubated at 37 °C in the presence of LGE_2 (10 μM) and [^{14}C]spermine (15 μM, 222 000 dpm). After 30 min, an aliquot was mixed with SDS loading buffer, boiled, and loaded onto a 7.5% SDS−PAGE gel. At the end of the electrophoresis, the proteins were stained with Coomassie Blue. The amount of radioactivity associated with the proteins was determined by autoradiography.

RESULTS

Characterization of the Adducts of PGHS-2 That Result from Reaction with Its Substrate. After PGHS-2 was incubated with [^{14}C]arachidonic acid and both the arachidonic acid and the products of the reaction were removed by a thorough dialysis against the reaction buffer, the amount of radioactivity associated with the protein was determined by C4 reversed phase HPLC coupled to a radioactivity detector on line (Figure 3). The radioactivity was associated with two peaks absorbing at 230 nm. Under these conditions, the UV peak corresponding to the radiolabeled protein had a retention time 2 min longer than that of the unreacted enzyme which also chromatographed in two peaks (data not shown).

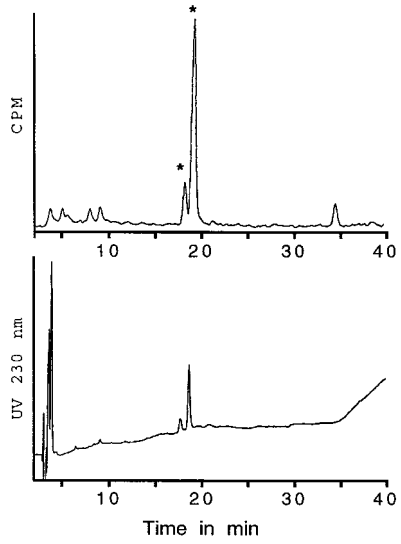

FIGURE 3: HPLC elution profile of radiolabeled PGHS-2. PGHS-2 was reconstituted in 100 mM sodium phosphate buffer (pH 7.5) and 500 μM phenol and incubated with [^{14}C]arachidonic acid at 37 °C for 30 min. After incubation, the enzyme was dialyzed and chromatographed on the C4 RP-HPLC system as described in Experimental Procedures. Under our experimental conditions, the radioactivity detector is delayed by 1 min compared to the UV detector. The asterisks correspond to the radioactivity associated with the enzyme.

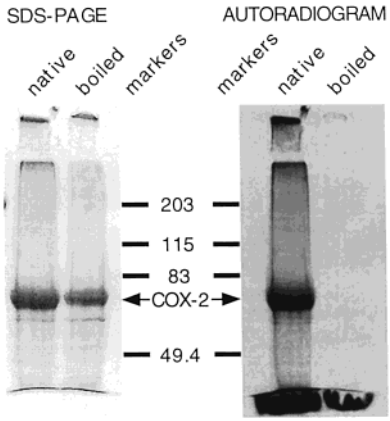

FIGURE 4: Dependence of PGHS-2 labeling on enzyme activity. Native PGHS-2 and boiled (for 10 min) PGHS-2 were reconstituted in 100 mM sodium phosphate buffer (pH 7.5) and 500 μM phenol and incubated with [^{14}C]arachidonic acid at 37 °C for 30 min. The proteins were then denatured by heat in the presence of SDS and loaded on a 10% SDS−PAGE gel. After the electrophoresis, the proteins were stained with Coomassie Blue and the gel was dried. The amount of radioactivity associated with the proteins was determined by autoradiography.

To further ascertain that the radioactivity was covalently bound to PGHS-2, the enzyme was incubated with [^{14}C]-arachidonic acid and analyzed under denaturing conditions by SDS−PAGE. The amount of radioactivity associated with the protein was determined by autoradiography after the proteins were stained with Coomassie Blue and the gel was dried (Figure 4). The autoradiogram shows clearly that the radioactivity comigrates with the protein when it is incubated with [^{14}C]arachidonic acid. On the contrary, when PGHS-2 was inactivated by boiling prior to incubation with its radioactive substrate, no radioactivity comigrated with the protein. After reaction of the enzyme with its substrate, some protein was detected at the interface between the stacking and separating gels, representing very high molecular weigh material; the radioactivity also was associated with it. This band was not present when the enzyme was boiled. This experiment rules out the possibility of any arachidonic acid being trapped in a noncovalent manner in the enzyme and demonstrates clearly the necessity of the catalytic activity to form the radioactive adducts.

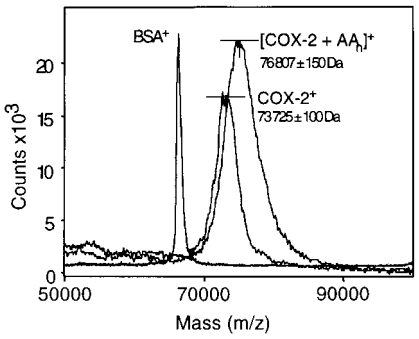

FIGURE 5: MALDI-TOF mass spectra of PGHS-2 before and after reaction with arachidonic acid. PGHS-2 was reconstituted in 100 mM sodium phosphate buffer (pH 7.5) and 500 μM phenol and incubated with [^{12}C]- or [^{14}C]arachidonic acid at 37 °C. At 0 and 2 min, an aliquot was taken and the reaction was stopped by mixing with an equal volume of acetonitrile. The samples (1 μL) were first deposited and allowed to dry on a conductive polyethylene membrane. The dried areas were washed with water prior to matrix deposition. The spectra were acquired in the linear mode under delayed extraction conditions using sinapinic acid as the matrix. The figure represents the comparative spectra of BSA, PGHS-2, and PGHS-2 after reaction with arachidonic acid for 2 min. The centroid peak values as well as the standard deviations have been determined by averaging five independent measurements.

To estimate the number of adducts formed on a molecule of PGHS-2, we incubated the enzyme with arachidonic acid and analyzed the adducted protein by MALDI-TOF mass spectrometry (Figure 5). The activity of the enzyme was assessed by incubation with [^{14}C]arachidonic acid and analysis of the radioactive products. Mass spectrometric analysis was carried out in a parallel experiment performed with unlabeled arachidonic acid. Figure 5 displays the mass profiles obtained for the singly charged molecular ion from PGHS-2 nonreacted and after incubation for 2 min with arachidonic acid. These two profiles are compared to the trace obtained with BSA under the same experimental conditions. It is apparent that the peak for nonreacted PGHS-2 is much broader than that for BSA, measured both at the base of the peaks and at half-height. Furthermore, the mass measured for PGHS-2 in its native state (73 725 ± 100) is higher than the mass calculated from its amino acid sequence (MW = 69 013; sequence from the Swiss-Prot database). These observations are consistent with the presence of multiple glycosylations of the native protein (*15*, *16*). The average mass of the peak after addition of arachidonic acid measured for the fraction sampled at 2 min is *m*/*z* 76 807 ± 150, and the peak is broader than that of the unreacted enzyme. The difference in average mass between the reacted and unreacted enzyme (3082) was divided by the average mass (333) of the two lysyl−LG adducts that were characterized, indicating that a mean of 9.3 molecules of LGE_2 are adducted per molecule of enzyme; given the width of the peak of the adducted enzyme, there is considerable heterogeneity in the number of adducts per

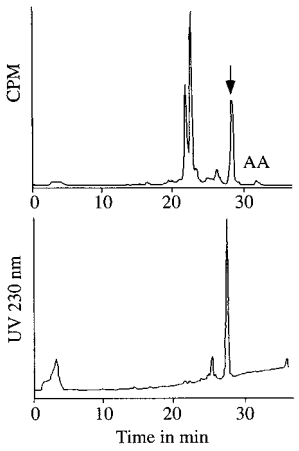

FIGURE 6: Chromatogram of the products of oxygenation of arachidonic acid by PGHS-2. PGHS-2 was reconstituted in 100 mM sodium phosphate buffer (pH 7.5) and 500 μM phenol and incubated with [^{14}C]arachidonic acid at 37 °C. At 40 s, 10 μL was mixed with sodium borohydride and injected onto the C4 VYDAC HPLC column as described in Experimental Procedures. The radioactivity and the UV absorbance at 230 nm were monitored. The arrow designates the radioactivity comigrating with the labeled enzyme. AA stands for residual arachidonic acid. The other radioactive peaks represent the nonreactive prostanoids derived from the hydrolysis of PGH_2. Under our experimental conditions, the radioactivity detector is delayed by 0.5 min from the UV detector.

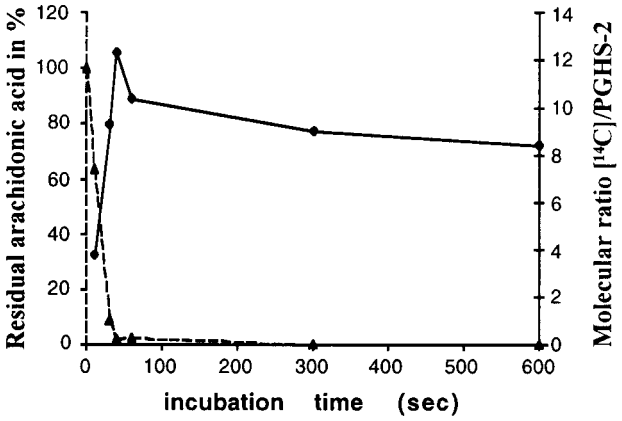

FIGURE 7: Kinetics of formation of covalent adducts on PGHS-2. PGHS-2 was reconstituted in 100 mM sodium phosphate buffer (pH 7.5) and 500 μM phenol and incubated with [^{14}C]arachidonic acid at 37 °C. At different times, 10 μL was mixed with sodium borohydride and injected onto a C4 VYDAC HPLC column as described in Experimental Procedures. The radioactivity and the UV absorbance at 230 nm were monitored. The amount of protein was determined using the area of the UV peak, and the oxygenation products were assessed by using the radioactive signals. The plain line represents the molecular ratio of the ^{14}C-labeled product coeluting with PGHS-2 and PGHS-2 (UV). The dashed line represents the residual arachidonic acid.

molecule of enzyme. This also confirms that the radioactivity associated with the protein as seen by reversed phase HPLC is covalently bound to it.

As previous investigations have demonstrated that approximately 18−22% of the PGH_2 rearranges to LG in aqueous solution (*1*, *2*), we examined the fraction of PGH_2 products that were adducted to PGHS-2. Figure 6 represents the radioactive profile and the UV chromatogram of the reaction mixture after incubation for 40 s when analyzed by C4 reversed phase HPLC. It indicates that in this experiment, 91.2% of the starting arachidonic acid (labeled AA in the trace) has been metabolized and that 17.3% of these oxygenated products are adducted to the protein (labeled with the arrow); that corresponds to the presence of 9.3 molecules of LGE_2 per molecule of enzyme (Experimental Procedures). The other major peaks observed in the beginning of the chromatogram correspond to the stable rearrangement products of PGH_2 in aqueous solution, including PGE_2, PGD_2, and $PGF_{2\alpha}$. Therefore, the number of products derived from arachidonic acid oxygenation by PGHS-2 that is adducted to the enzyme corresponds to the number of covalent arachidonic acid-derived adducts per molecule of enzyme calculated from the MALDI experiment.

To assess the rate of formation of the covalent adducts with the enzyme, we performed the same labeling experiment and analyzed the radioactive products as a function of time (Figure 7). The results showed, in accordance with the previous finding (*17*, *18*), that the enzymatic reaction is over in 30 s and that the formation of adducts begins within that time and is completed within the next minute. It also showed that the molecular ratio of LGE_2 to PGHS-2 changes little over a period of time from 30 s to 10 min. This implies that the observations made after long incubations reflect an event that takes place very early. Interestingly, there was a loss in the level of protein over the time that was reflected by a decrease in the area of the UV peak after incubation for 5 min; this may represent the formation of cross-linked species (*6*) that are trapped on the guard column.

Two adducts derived from the reaction of lysine and LGE_2 or PGH_2 previously have been characterized (*2*, *8*). The lysyl−LG Schiff base can be isolated following its reduction with sodium borohydride, during which it also undergoes dehydration to the reduced lysyl−anhydroLG Schiff base adduct (*m*/*z* 467.4). On the basis of the knowledge of the structure of these predominant lysyl−levuglandin adducts, it was possible to demonstrate that these adducts were formed on the PGHS enzymes as a consequence of the oxygenation of arachidonic acid. To accomplish this, standards for the reduced lysyl−anhydrolevuglandin Schiff base and for the lactam adducts were prepared by reaction of synthetic LGE_2 with [$^{13}C_6$,^{3}H]lysine. After PGHS-2 was incubated with [^{14}C]-arachidonic acid, it was enzymatically digested to yield single amino acids. The standard was added, isolated from the proteolysis mixture, and analyzed by liquid chromatography−tandem mass spectrometry (LC−MS/MS). As depicted in Figure 8 (left panels), the daughter ion (*m*/*z* 321.4) of the reduced lysyl−anhydrolevuglandin Schiff base adduct from PGHS-2 {*m*/*z* 467.4; [(M + 1) − H_2O]} is clearly demonstrated, coeluting with the internal standard. The daughter ion (*m*/*z* 84.1) of the lactam−levuglandin adduct (*m*/*z* 479.4; right panels) is also evident. The shapes of the signals conform to those demonstrated with the reaction products of lysine and PGH_2 or synthetic LGE_2 (*2*, *8*). The dual peak at *m*/*z* 321.4 for the reduced lysyl−anhydrolevuglandin Schiff base internal standard is presumed to result from the two isomers of the 15-hydroxyls present in synthetic LGE_2, whereas the single peak at *m*/*z* 321.4 from the PGHS-2 digest is consistent with its formation from 15(*S*)-PGH_2 via 15(*S*)-LGE_2. On the other hand, the lactam internal standard at *m*/*z* 89.1 contains four enantiomers due to the racemic nature of positions 9 and 15: the 9(*R*),15(*R*)-, the 9(*S*),15(*S*)-, the

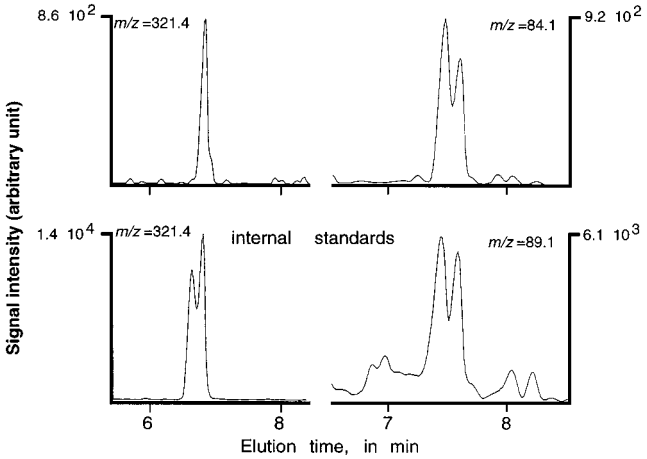

FIGURE 8: Arachidonic acid-derived adducts from PGHS-2. After reaction with arachidonic acid (Experimental Procedures), PGHS-2 was digested to single amino acids by step digestion with pronase and aminopeptidase M. The adducts were purified by chromatography on an Oasis cartridge followed by reverse phase HPLC, and then analyzed by LC−ESI-MS/MS. Selected reaction monitoring chromatograms for the daughter ions of the reduced Schiff base (*m*/*z* 321.4) and for the lactam (*m*/*z* 84.1) adducts are shown.

9(*S*),15(*R*)-, and the 9(*R*),15(*S*)-lysyl−LG lactam adducts. The first two and the last two are diastereoisomers, and the four enantiomers are resolved in a dual peak by reversed phase HPLC. In the same way, the lactam adduct obtained from 15(*S*)-PGH_2 gives the two enantiomers, 9(*R*),15(*S*)- and 9(*S*),15(*S*)-lysyl−LG lactam adducts, that are separated in a dual peak by reversed phase HPLC. The same adducts were characterized when the experiment was carried out with PGHS-1 (data not shown). This establishes the molecular identity of two of the adducts of both cyclooxygenases derived from the oxygenation of arachidonic acid, and provides information that enhances the capability for identifying adducted proteins in biological systems.

Formation of Adducts with Other Proteins. To assess whether reactive products of arachidonic acid oxygenation by PGHS-2 would adduct to lysine-rich proteins such as histones and ubiquitin, we co-incubated PGHS-2 with [^{14}C]-arachidonic acid and each protein (Figure 9). After electrophoresis on denaturing SDS−PAGE under reducing conditions to separate the different proteins, the gels were dried and the amount of radioactivity was determined by autoradiography. As shown in Figure 9, histones (panel A) and ubiquitin (panel B) comigrate with the radioactivity when the arachidonic acid is oxidized by PGHS-2 (left lanes). If the cyclooxygenase is not present, there is no association of radioactivity with the proteins (right lanes).

Reactivity of the Protein Adducts. A high-molecular weight species of PGHS-2 that did not enter the SDS−PAGE separating gel was formed after oxygenation of arachidonic acid (Figure 4). This suggests an aggregation of PGHS-2 molecules that are cross-linked as a consequence of adduct formation. To obtain more direct evidence that the adducts of PGHS-2 are reactive and capable of initiating intermolecular cross-linking, we incubated PGHS-2 with unlabeled arachidonic acid and the polyamine [^{14}C]spermine. The amount of radioactivity associated with the enzyme was monitored by SDS−PAGE. The same experiment was carried out with synthetic LGE_2 in place of PGHS-2. As shown in Figure 10, radioactivity was associated with the proteins, and this was dependent upon the presence of LGE_2 or PGHS-2. It establishes that LGE_2 can cross-link spermine to proteins and indicates that spermine, a polyamine that is abundant in both the cytosol and nucleus, could be a potential marker for the PGHS-2-dependent formation of adducts in cells.

DISCUSSION

These findings characterize the adducts formed between oxygenated metabolites of arachidonic acid and PGHS-1 and PGHS-2. As previously has been demonstrated for PGHS-1 (*3*), following oxygenation of [^{14}C]arachidonic acid by PGHS-2, the enzyme comigrates with the radiolabel on SDS−PAGE, and coelutes with the radiolabel on reversed phase chromatography. Moreover, such comigration is prevented if the protein has been inactivated by boiling prior to the incubation with the radiolabeled substrate, indicating that the formation of adducts is a consequence of arachidonate oxygenation. The formation of arachidonate-derived adducts of PGHS-2 also is indicated by an increase in the mass of the reacted enzyme that is observed by MALDI-TOF mass spectrometric analysis. The magnitude and rate of adduct formation indicate that a highly reactive product is formed by the cyclooxygenases and that it has reacted with the enzyme within 1 min.

The fraction of oxygenated arachidonic acid adducted to the cyclooxygenase was assessed in two ways in the study presented here. HPLC of the reaction products derived from [^{14}C]arachidonic acid demonstrated that 17.3% of the products of the reaction are present as adducts of the cyclooxygenase (Figure 6); this corresponds to ~9 adducts per molecule of PGHS-2. This value is relatively constant for a period of time between 30 s and 10 min (Figure 7). This is consistent with the average of 9.3 ± 0.4 molecules of levuglandin adducted to each molecule of the enzyme determined by MALDI-TOF after incubation for 2 min. Under the conditions of this experiment, 49.7 molecules of arachidonic acid were oxygenated per molecule of enzyme (16.52 nmol of products generated by 332.5 pmol of enzyme). Therefore, the adducted levuglandins account for 18.7% of the reaction products. Both of these measurements are considered approximations inasmuch as they do not account for the cyclooxygenase molecules that have been cross-linked to form multimers. Salomon and colleagues estimated that levuglandins constitute 22% of the products formed from PGH_2 in aqueous solution based on analysis with nuclear magnetic resonance spectroscopy (*1*), and we found that 18% of the PGH_2 was transformed to levuglandins after 30 min utilizing gas chromatography−mass spectrometry for analysis (*2*). Thus, approximately 18−22% of the PGH_2 generated by PGHS-2 has been found to rearrange into levuglandins, and we find that 17.3−18.7% of the products of the oxygenation of arachidonic acid are found to be adducted to the enzyme. As PGH_2 is the only product of the catalytic oxygenation of arachidonic acid by PGHS-2, and as no covalent adduct is formed if the enzyme is omitted or denatured, we conclude that almost all the levuglandins generated by spontaneous rearrangement of PGH_2 in buffer [18−22% (*1*, *2*)] form covalent adducts with the enzyme. This is consistent with the high degree of reactivity of these potent electrophiles.

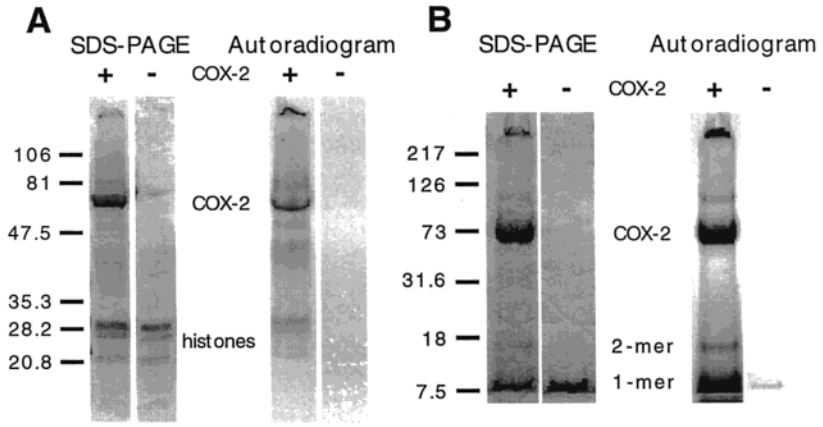

FIGURE 9: Arachidonic acid-derived adducts of histones and ubiquitin. After co-incubation at 37 °C of PGHS-2, [^{14}C]arachidonic acid, and histone types II and III from calf thymus (A) and ubiquitin (B) for 30 min, the proteins were denatured at 70 °C in the presence of LDS and separated by electrophoresis using the NuPAGE MOPS running buffer. At the end of the electrophoresis, the proteins were stained with Coomassie Blue and the gel was dried under vacuum. The amount of radioactivity associated with the proteins was determined by autoradiography.

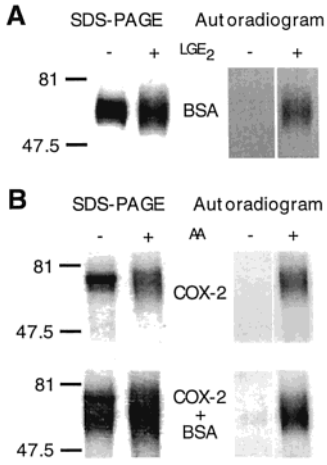

FIGURE 10: Cross-linking of albumin and spermine by LGE_2. After co-incubation at 37 °C of BSA with [^{14}C]spermine and synthetic LGE_2 (A) or PGHS-2 and cold arachidonic acid (B) for 30 min, the proteins were denatured at 70 °C in the presence of LDS and separated by electrophoresis using the NuPAGE MOPS running buffer. At the end of the electrophoresis, the proteins were stained with Coomassie Blue. The amount of radioactivity associated with the proteins was determined by autoradiography.

The average number of adducts (9−9.3) per molecule of enzyme was determined to permit calculation of the fraction of the oxygenated products of arachidonic acid that is adducted to the PGHS; this is the number of adducts that is relevant only to the conditions of these experiments (e.g., substrate and enzyme concentrations) and does not reflect the maximum number of adducts that could be formed with saturating concentrations of levuglandins. Indeed, the broadening of the PGHS peak on MALDI-TOF that results from the oxygenation of arachidonic acid reflects a considerable heterogeneity of the number of adducts per molecule of enzyme, indicating that many more than 9 lysine sites can be adducted.

In these investigations of the extent of adduct formation on PGHS-2, the enzyme is the only target for reaction with the LGs. However, the electrophilic reactivity of sites on the levuglandin molecules leads to the formation of covalent bonds not only with the cyclooxygenases but also with other biological nucleophiles in solution with the products of the oxygenation of arachidonic acid [Figure 9 (*3*)]. Thus, in cells and in vivo, other proteins will compete with the PGHSs for LG adduction; in platelets, multiple proteins in addition to PGHS-1 are adducted by oxygenated products of arachidonic acid (*5*). Knowledge of the chemical nature of the arachidonic acid-derived adducts of the PGHSs predicts that amines also will adduct with the LGs as, for example, Tris buffer is known to do (*19*); this may account for the lower proportion of adduct bound to the enzyme (*3*) when oxygenation of arachidonic acid is carried out in Tris buffer that competes with the enzyme for adduct formation. To avoid such competition, we performed all our labeling experiments in sodium phosphate buffer.

It is noted that LGE_2 forms covalent adducts with proteins (*8*) (Figure 7) at a higher rate than it does with free lysine (*2*). This indicates that the tertiary structure of the proteins might influence the rate of the reaction with the lysine residues (*20*).

Characterization of the structure of the adducts was based on the hypothesis that they were derived from the levuglandins. To develop an analytical strategy for determining whether adducts of the cyclooxygenases and other proteins were derived from levuglandins, we characterized the structures of adducts of synthetic LGE_2 with lysine, and demonstrated the formation of the lysyl−LG Schiff base (*2*) as well as oxidative products of the lysyl−LG pyrrole, the lysyl−LG lactam, and the lysyl−LG hydroxylactam (*8*) (Figure 2). All of these products also could be formed by incubation of PGH_2 with lysine, validating this as a tangible pathway of PGH_2 rearrangement.

We here report evidence that a levuglandin is adducted to the PGH-synthases as a consequence of the oxygenation of arachidonic acid by the enzymes. After protease digestion of reacted PGHS-2 that has been treated with a reducing agent, the reduced lysyl−LG Schiff base has been isolated and characterized by cochromatography with a ^{13}C-labeled standard and with LC−MS/MS, based on both the molecular ion {*m*/*z* 467.4; [M + 1 − H_2O]} and the characteristic daughter ion (*m*/*z* 321.4). The lysyl−LG lactam adduct also is identified, with its molecular ion (*m*/*z* 479.4) and the daughter ion at *m*/*z* 84.1. Similar evidence indicates that

lysyl−LG Schiff base and lactam adducts of PGHS-1 are formed following the oxygenation of arachidonic acid.

Importantly, the initial lysyl−levuglandin adducts themselves are also highly reactive; the Schiff base adduct with lysine can undergo nucleophilic attack (*21*), and the oxidation of the pyrrole adducts generates sites of electrophilic reactivity (*22*, *23*). Thus, lysyl−LG adducts can form covalent bonds with other nucleophiles, leading to intermolecular cross-linking. Such cross-linking has been demonstrated for adducts derived from synthetic levuglandin E_2; e.g., 23% of the albumin is cross-linked after 30 min when it is co-incubated with LGE_2 (*6*). Time-dependent formation of a cyclooxygenase-derived protein that will not enter into or migrate on PAGE gels probably results from formation of cyclooxygenase multimers. Also, loss of the protein over time after incubation with arachidonic acid was reflected by a decrease in the magnitude of the UV signal on C4 HPLC corresponding to the enzyme. The reactivity of the initial lysyl−LG adducts is clearly demonstrated by the evidence presented here that spermine will react covalently with PGHS-2 molecules that have been adducted by oxygenated products of arachidonic acid (Figure 10).

In summary, the lysyl−LG Schiff base and the pyrrole-derived lysyl−LG lactam have been characterized as adducts of the cyclooxygenases that are formed from PGH_2 via the levuglandin pathway. This provides a molecular basis for lipid modification of proteins and other biologic nucleophiles. Whereas the present knowledge of covalently attached lipid moieties cannot predict the effects of eicosanoylation on the function and localization of a protein, it is known that lipid modification can affect the localization (*24*), degradation, or function of proteins. Given the high degree of reactivity of the levuglandins, the biological significance of their formation will be a function of the rate of catalytic disposition of PGH_2 by prostanoid synthases in different normal and pathologic cells, and of the nucleophiles that are the targets of monomolecular adduct formation or intermolecular cross-linking. This approach to characterizing the lysyl−LG Schiff base and lysyl−LG lactam adducts of PGHS-2 now provides a means for determining whether this pathway of arachidonic acid metabolism exists in cellular environments in which PGHSs are expressed.

ACKNOWLEDGMENT

We gratefully acknowledge the capable technical assistance of Elizabeth Shipp, Jiemin Wang, and Gwenn Sobo.

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BI002629K