Inactivation of Prostaglandin Endoperoxide Synthase by Acylating Derivatives of Indomethacin†

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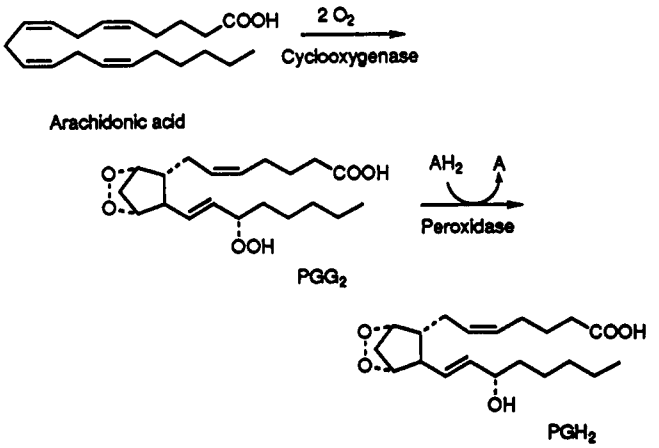
ABSTRACT: Derivatives of the potent antiinflammatory agent and cyclooxygenase inhibitor indomethacin were synthesized in which the carboxylic acid moiety was converted into reactive acylating agents. Indomethacin imidazole (indomethacin-IM) and indomethacin *N*-hydroxysuccinimide (indomethacin-NHS) inactivated both the cyclooxygenase and peroxidase activities when incubated with the apo form of purified prostaglandin endoperoxide synthase (PGH synthase) at a stoichiometry of 1:1. Treatment of the inactivated enzyme with hydroxylamine at neutral pH led to recovery of all peroxidase and about 50% of the cyclooxygenase activity. Hydroxylamine did not regenerate the cyclooxygenase activity of indomethacin-inactivated protein. Reconstitution of the apoprotein with heme protected against inactivation by indomethacin-NHS. Visible spectroscopy established that indomethacin-NHS-inactivated apoenzyme had a reduced capacity to bind heme. Indomethacin-NHS also substantially protected the apoenzyme from cleavage at the trypsin-sensitive Arg^{277} site. Incubation of [2-^{14}C]indomethacin-NHS with PGH synthase led to incorporation of radioactivity into the protein, but no adduct was detected by reversed-phase HPLC, suggesting it was unstable to the chromatographic conditions. Incubation of indomethacin-NHS with apoprotein followed by HPLC analysis led to the formation of greater amounts of the hydrolysis product indomethacin than did similar treatment of holoprotein. The results suggest that indomethacin-IM and indomethacin-NHS covalently and selectively label PGH synthase near the heme binding site, leading to loss of both catalytic activities of the enzyme.

Prostaglandin endoperoxide (PGH)[1] synthase catalyzes the first two steps of the prostaglandin biosynthetic pathway (Smith & Marnett, 1991). Its cyclooxygenase activity oxygenates arachidonic acid to the hydroperoxy endoperoxide PGG_2, and its peroxidase activity reduces PGG_2 to the alcohol PGH_2 in the presence of a reducing substrate (eq 1) (Hamberg et al., 1974; Nugteren & Hazelhof, 1973). The holoenzyme is a homodimer of 70-kDa subunits containing one heme per subunit (Van Der Ouderaa et al., 1977; Ruf et al., 1984); each monomer appears to catalyze both activities (Ruf et al., 1992). PGH synthase is inactivated in vitro and in vivo by a variety of agents known as nonsteroidal antiinflammatory drugs (NSAIDs). These compounds, which include aspirin, flurbiprofen, and indomethacin, inhibit the cyclooxygenase but not the peroxidase activity of the enzyme (Robinson & Vane, 1974; Rome & Lands, 1975). Aspirin covalently modifies the PGH synthase protein by transferring its acetyl group to Ser^{530} (Roth et al., 1975; Van Der Ouderaa et al., 1980; Rome et al., 1976).[2] Indomethacin and flurbiprofen associate tightly but noncovalently with the protein at a stoichiometry of 1 mol/mol of synthase dimer (Kulmacz & Lands, 1985). The ability of indomethacin to protect apoPGH synthase from trypsin cleavage at Arg^{277} suggests it induces a conformational change when it binds to the protein (Kulmacz & Wu, 1989).



Conversion of the carboxylic acid moiety of indomethacin and related molecules to reactive acylating derivatives could be useful for covalent modification of PGH synthase near its drug binding site. We and others have found that *N*-acetylimidazole (NAI) inactivates the cyclooxygenase and peroxidase activities of PGH synthase when it reacts with apo- but not holoenzyme (Wells & Marnett, 1992; Scherer et al., 1992). Inhibition is reversed by hydroxylamine treatment under neutral conditions, suggesting inactivation results from modification of tyrosine, histidine, or cysteine residues. Peptide mapping of the [^{3}H]NAI-acetylated protein reveals a number of potential acetylation sites. The large excess of NAI (100–1000-fold) needed to inactivate PGH synthase leads to substantial nonspecific acetylation. The imidazole derivative of indomethacin (indomethacin-IM) should be a more selective inactivator of PGH synthase than NAI because of the high affinity of PGH synthase for the (*p*-chlorobenzoyl)indole

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[1] Abbreviations and trivial names: PGH synthase, prostaglandin endoperoxide synthase (EC 1.14.99.1); NSAID, nonsteroidal antiinflammatory drug; NAI, *N*-acetylimidazole; indomethacin, 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid; indomethacin-IM, 1-(4-chlorobenzoyl)-3-[2-(1*H*-imidazol-1-yl)-2-oxoethyl]-5-methoxy-2-methyl-1*H*-indole; indomethacin-NHS, *N*-succinimidyl 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetate; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; EI, electron impact.

[2] The numbering of the amino acids begins with the translation start site (DeWitt & Smith, 1988).

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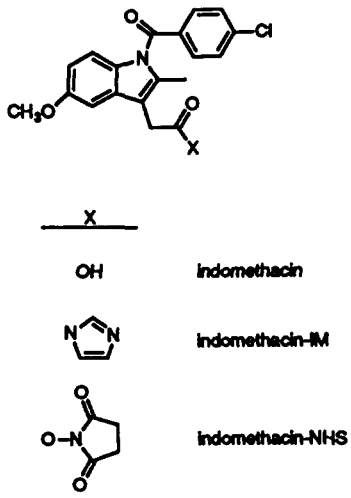

FIGURE 1: Structures of indomethacin, indomethacin-IM, and indomethacin-NHS.

moiety. We report herein that both indomethacin-IM and the indomethacin *N*-hydroxysuccinimide ester (indomethacin-NHS) (Figure 1) are potent inactivators of PGH synthase that selectively and covalently modify the protein. Inactivation is specific to apoprotein and appears to result from prevention of heme binding to the active site.

MATERIALS AND METHODS

Materials

Indomethacin, *N*-hydroxysuccinimide, hematin, and trypsin (TPCK treated, type XIII) were purchased from Sigma (St Louis, MO). 1,1'-Carbonyldiimidazole was from Aldrich (Milwaukee, WI), and arachidonic acid was from Nu Check Prep (Elysian, MN). [2-^{14}C]Indomethacin (37.7 mCi mmol^{-1}) was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade or better. NMR spectra were taken on a Bruker AC300 300-MHz spectrometer. Electron impact mass spectra were recorded on a Delsi Nermag R10-10 C spectrometer, and fast atom bombardment mass spectra were recorded on a VG70-250HF spectrometer.

PGH synthase was purified from ram seminal vesicles (Marnett et al., 1984), and apoenzyme was prepared by gel filtration as previously described (Odenwaller et al., 1990). Apoprotein was desalted on a Sephadex G-25 column eluted with 10 mM Tris-HCl (pH 7.5). It was at least 99% apo, and its specific activity ranged from 25 to 74 μmol of O_2 (mg of protein min)$^{-1}$ when assayed in the presence of excess hematin. Holoenzyme was reconstituted by addition to apoenzyme of 1 or 2 equiv of hematin from a 500 μM stock solution in DMSO.

Preparation of Indomethacin-IM. Indomethacin (1.78 g, 5 mmol) and 1,1'-carbonyldiimidazole (3.6 g, 21 mmol) were dissolved in dry THF (15 mL) in a 25-mL round-bottom flask, and the reaction was stirred for 1 h at room temperature under nitrogen. Imidazole was filtered off, and anhydrous ether (15 mL) was added to the filtrate. The product crystallized and was collected by filtration. It was washed with ether, dried overnight in a desiccator under vacuum, and analyzed by thin-layer chromatography (TLC, silica gel, eluted with ethyl acetate). The material exhibited a single spot at R_f = 0.46. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.42 (s, 3 H), 3.80 (s, 3 H), 4.22 (s, 2 H), 6.68 (dd, J = 2.5, 9 Hz, 1 H), 6.84 (d, J = 9 Hz, 1 H), 6.86 (d, J = 2.5 Hz, 1 H), 7.13 (m, 1 H), 7.47 (d, J = 8.4 Hz, 2 H), 7.56 (m, 1 H), 7.66 (d, J = 8.4 Hz, 2 H), 8.27 (s, 1 H) ppm; EI MS M^+ = 407.

Preparation of Indomethacin-NHS. Indomethacin (90 mg, 0.25 nmol) was dissolved in 75% DMF in water (10 mL, pH 5) in a 50-mL round-bottom flask. *N*-Hydroxysuccinimide (57.5 mg, 0.5 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (479 mg, 2.5 mmol) were added and the reaction was stirred at room temperature. Separation of the product was observed, and after 90 min, water was added to induce complete precipitation. The precipitate was filtered and washed with water and then was dried overnight in a desiccator under vacuum. The product was obtained in 77% yield and chromatographed as a single spot on TLC (silica gel eluted with ethyl acetate, R_f = 0.87). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.36 (s, 3 H), 2.81 (s, 4 H), 3.84 (s, 3 H), 3.95 (s, 2 H), 6.67 (dd, J = 2.5, 9 Hz, 1 H), 6.89 (d, J = 9 Hz, 1 H), 6.95 (d, J = 2.5 Hz, 1 H), 7.45 (d, J = 8.5 Hz, 2 H), 7.65 (d, J = 8.5 Hz, 2 H) ppm. The product contained 4% water. EI MS M^+ = 454.

Preparation of [2-^{14}C]Indomethacin-NHS. [2-^{14}C]Indomethacin (2.37 mg, 6.6 μmol, 37.7 mCi mmol^{-1}) was diluted with cold indomethacin (7.6 mg, 21.2 μmol). To the vial containing the radiolabeled material were added 1 mL of 75% DMF in water (pH 5), *N*-hydroxysuccinimide (6.4 mg, 55 μmol), and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (53 mg, 270 μmol). The reaction was stirred for 30 min at room temperature and then worked up as described above. The product, obtained in 54% yield, had an average specific activity of 6.4 mCi mmol^{-1}. Its radiochemical purity was 88% by TLC analysis. Only one radiolabeled peak was observed on HPLC (Vydac C_4 column eluted as described under chromatography conditions in Methods).

Methods

Cyclooxygenase Assay. Cyclooxygenase activity was measured at 37 °C with a Gilson Model 5/6H oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) fitted with a 1.3-mL water-jacketed cell. Enzyme aliquots were added to 100 mM Tris-HCl at pH 8 containing 500 μM phenol and 1 μM hematin. Oxygen uptake was initiated by addition of 100 μM arachidonic acid, and the initial reaction velocity was determined from the linear portion of the O_2 uptake curve.

Peroxidase Assay. Assays were performed on a Shimadzu UV 160U by measuring the oxidation of guaiacol at 436 nm or *N,N,N',N'*-tetramethyl-1,4-phenylenediamine (TMPD) at 610 nm and calculating the slope of the initial linear portion of the curve. Enzyme aliquots were added to 100 mM Tris-HCl (pH 8) containing 1 μM hematin and 400 μM H_2O_2 in 1-mL disposable cuvettes followed by addition of 500 μM guaiacol or TMPD.

PGH Synthase Inactivation by Indomethacin-IM or Indomethacin-NHS and Reactivation by Hydroxylamine. Apo- or holoenzyme (17 μM) was incubated on ice in 10 mM Tris-HCl (pH 7.5) with 1 or 2 equiv of the compound of interest added in 4 μL of DMF. The time course for inactivation was determined by testing aliquots for cyclooxygenase and peroxidase activity. After inactivation of the enzyme, hydroxylamine was added from a 2 M solution in 10 mM Tris-HCl (pH 7.5) to a final concentration of 120–240 mM as specified.

Trypsin Digestion and Gel Electrophoresis. Inactivated apoenzyme was treated with 2% w/w trypsin (prepared in 1 mM HCl). After a 40-min digestion at room temperature, 20 equiv of trypsin inhibitor (Sigma, type II-O) was added. A 20-μg enzyme aliquot was loaded on a 10% polyacrylamide gel, and electrophoresis was performed according to the procedure of Laemmli et al. (1970).

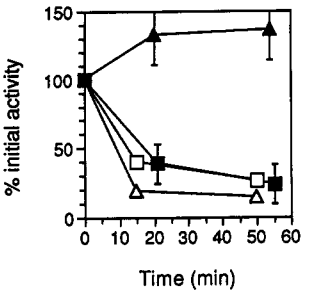

FIGURE 2: Time course for inactivation of apoenzyme by indomethacin or indomethacin-IM. ApoPGH synthase (20 μM, 180 μg) was incubated with 2 equiv of indomethacin or indomethacin-IM at 0 °C in 10 mM Tris-HCl (pH 7.5). At various time points, 15-μg aliquots were tested for cyclooxygenase (△, indomethacin; □, indomethacin-IM) and peroxidase (▲, indomethacin; ■, indomethacin-IM) activity as described in Materials and Methods. Results are plotted as the percentage of initial activities. The peroxidase was assayed in triplicate at each time point with the indicated standard deviation.

Chromatography Conditions for the Modified Enzyme. The modified protein (60 μg) was injected on a Vydac C_4 column (0.46 × 25 cm) eluted with a solvent system of A = 0.1% TFA and B = 0.1% TFA in 80% acetonitrile and a linear gradient from 42% to 58% acetonitrile in 30 min (flow rate 1 mL min^{-1}). The HPLC was connected to a diode array UV detector and integrator (Hewlett-Packard Chemstation 1040A and 9153C) and the absorbance monitored at 230, 260, and 315 nm. Radiolabeled samples were injected on the same HPLC system connected to a Varian 2050 UV detector (λ = 230 nm) and to a Radiomatic Flo-one βeta radioactive flow detector.

Absorption Spectra of Free and Bound Heme. The UV–visible spectrum of free or PGH synthase-bound heme was measured from 350 to 450 nm on a 8452A Hewlett-Packard diode array spectrophotometer. The 1-mL disposable cuvette contained 1.5 μM free heme or 1.5 μM (native or inactivated) apoenzyme reconstituted with 1 equiv of hematin, in 118 mM Tris-HCl (pH 8).

Spectrophotometric Titrations. Heme titration of indomethacin- or indomethacin-NHS-treated apoenzyme was performed in 1-mL disposable cuvettes in a Shimadzu UV 160U spectrophotometer. Aliquots of a 100 μM solution of hematin in DMSO/50 mM sodium phosphate, pH 7.5 (ratio 1:4), were added to the sample cuvette, which contained 2 μM protein in 50 mM phosphate (pH 7.5). An identical amount of hematin was added to the reference cuvette, which contained buffer alone. The absorption spectrum was scanned from 350 to 500 nm. The increase in absorbance at 411 nm was calculated from the difference spectrum.

RESULTS

Inactivation of PGH Synthase and Reactivation by Hydroxylamine. Treatment of apoPGH synthase with indomethacin-IM led to loss of both cyclooxygenase and peroxidase activities at comparable rates (Figure 2). About 30% of the initial activities remained after a 50-min incubation with 2 equiv of indomethacin-IM. For comparison, apoenzyme was treated with the same concentration of indomethacin. The cyclooxygenase activity decreased to the same extent as observed with indomethacin-IM, but the peroxidase activity was not inhibited (Figure 2).

Inhibition of cyclooxygenase and peroxidase was not reversed by overnight dialysis of indomethacin-IM-inactivated apoenzyme against the reaction buffer [10 mM Tris-HCl (pH 7.5)]. However, both activities of the inactivated apoenzyme were restored upon treatment with 240 mM hydroxylamine at pH 7.5 for 30 min. All of the peroxidase activity and about half of the cyclooxygenase activity were recovered as shown in Figure 3. This is consistent with a reversal of acylation and formation of the corresponding indomethacin hydroxamate which is a cyclooxygenase inhibitor but a less potent one than indomethacin (Flynn et al., 1990). The cyclooxygenase activity of indomethacin-inactivated enzyme was not restored by dialysis or hydroxylamine treatment.

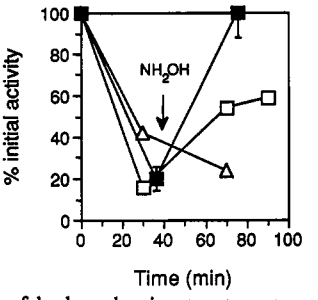

FIGURE 3: Effect of hydroxylamine treatment on cyclooxygenase and peroxidase activities of indomethacin-IM-inactivated apoPGH synthase. Apoenzyme (17 μM, 150 μg) was treated with 2 equiv of indomethacin or indomethacin-IM at 0 °C in 10 mM Tris-HCl (pH 7.5). After 40 min, 240 mM hydroxylamine was added as indicated on the graph. At various time points, 15 μg of enzyme was removed and tested for cyclooxygenase (△, indomethacin; □, indomethacin-IM) and peroxidase (■, indomethacin-IM) activity as described in Materials and Methods. The results are plotted as percentage of initial activity. The peroxidase activity was assayed in triplicate at each time point with the indicated standard deviation.

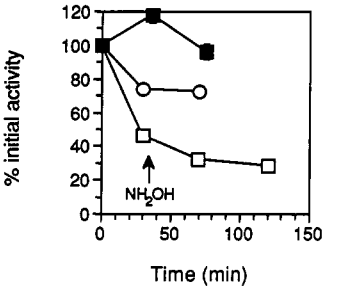

FIGURE 4: Inactivation of holoenzyme by indomethacin-IM or indomethacin-NHS. HoloPGH synthase (17 μM, 150 μg) was treated with 2 equiv of indomethacin-IM or indomethacin-NHS at 0 °C in 10 mM Tris-HCl (pH 7.5). As indicated on the graph, 240 mM hydroxylamine was added to both samples. At various time points, 15-μg aliquots were tested for cyclooxygenase (□, indomethacin-IM; ○, indomethacin-NHS) and peroxidase (■, indomethacin-IM) activity as described in Materials and Methods. The results are plotted as percentage of initial activity. The peroxidase activity was assayed in triplicate at each time point with the indicated standard deviation.

In order to study the effect of the heme prosthetic group on inactivation, reconstituted holoenzyme was treated with 2 equiv of indomethacin-IM. Cyclooxygenase activity decreased with time, but the peroxidase activity did not (Figure 4). Furthermore, upon treatment with 240 mM hydroxylamine for 30 min, no cyclooxygenase activity was recovered. The similarity of these observations to those made with indomethacin suggested that indomethacin-IM hydrolyzed in the reaction buffer to indomethacin which inactivated the cyclooxygenase activity of the holoenzyme. In order to circumvent this problem, we synthesized the *N*-hydroxysuccinimide ester (indomethacin-NHS); *N*-hydroxysuccinimide esters of carboxylic acids are relatively stable to hydrolysis in neutral aqueous solutions (Wong, 1991).

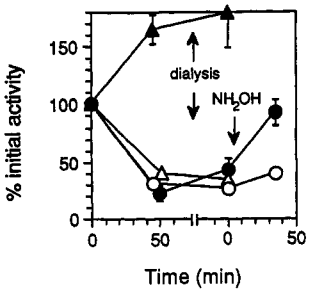

FIGURE 5: Effect of hydroxylamine treatment on cyclooxygenase and peroxidase activities for indomethacin-NHS-inactivated apoPGH synthase. To apoenzyme (16 μM, 150 or 300 μg) in 10 mM Tris-HCl (pH 7.5) was added 2 equiv of indomethacin or indomethacin-NHS at 0 °C. Inhibition of the enzyme was followed by removing 15-μg aliquots for cyclooxygenase (△, indomethacin; ○, indomethacin-NHS) and peroxidase activity (▲, indomethacin; ●, indomethacin-NHS) as described in Materials and Methods. After 1 h, the samples were dialyzed overnight against 10 mM Tris-HCl (pH 7.5) at 4 °C and 120 mM hydroxylamine was added as indicated. The results are plotted as percentage of initial activity. The peroxidase activity was assayed in triplicate at each time point with the indicated standard deviation.

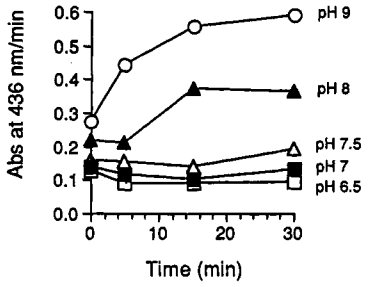

FIGURE 6: pH Dependence of the spontaneous recovery in peroxidase activity of indomethacin-NHS-treated apoPGH synthase. Apoenzyme (20 μM, 500 μg) was treated with 3 equiv of indomethacin-NHS for 90 min at 0 °C in 10 mM Tris-HCl (pH 7.5). At that point, 27% of the initial peroxidase activity remained. Aliquots of the modified enzyme were then incubated at room temperature in 1-mL disposable cuvettes containing 50 mM phosphate buffer at pH 6.5–9. Peroxidase activity was assayed using guaiacol as a reducing substrate as described in Materials and Methods.

Indomethacin-NHS exhibited similar inhibitory properties to indomethacin-IM when incubated with apoenzyme (Figure 5). Indomethacin-NHS inactivated both cyclooxygenase and peroxidase activities to comparable extents after 50 min, and hydroxylamine treatment partially restored both activities. Holoenzyme treated with indomethacin-NHS lost little cyclooxygenase activity (<30%) and no peroxidase activity after 70 min (Figure 4). Thus, it appeared that indomethacin-NHS was not able to bind covalently to holoenzyme. Identical results were obtained when this experiment was repeated in 100 mM sodium phosphate buffer (pH 7.8), which eliminated the possibility that the lack of inhibition was due to reaction with Tris buffer. These experiments indicated that the presence of the heme prosthetic group protected PGH synthase from inactivation from indomethacin-NHS. Since indomethacin-NHS was more stable toward hydrolysis than indomethacin-IM, we used the former reagent for the rest of our experiments.

The pH dependence of the spontaneous recovery of the peroxidase activity of indomethacin-NHS-treated apoenzyme was investigated. As shown in Figure 6, incubation of indomethacin-NHS-inactivated apoenzyme at room temperature in 50 mM sodium phosphate buffers from pH 6.5 to 9 led to recovery of peroxidase activity that was more rapid at higher pH's.

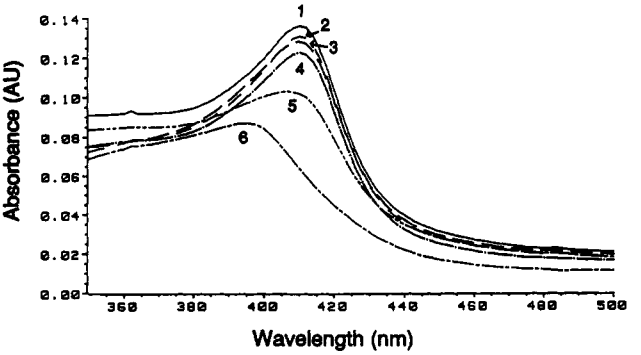

FIGURE 7: Effect of indomethacin-NHS on holoenzyme reconstitution. The absorption spectrum of free or PGH synthase-bound heme (1.5 μM) was measured as described in Materials and Methods. Apoenzyme (1.5 μM) was treated with 2 equiv of inhibitor for 30 min as indicated and then was reconstituted with 1 equiv of hematin. Each spectrum was zeroed at 700 nm to compensate for differences in absorbance at this wavelength. Spectrum 1, apoenzyme inactivated by 2 equiv of indomethacin; 2, holoenzyme; 3, apoenzyme inactivated by indomethacin-NHS followed by reactivation with 180 mM hydroxylamine for 1 h; 4, holoenzyme treated with 2 equiv of indomethacin-NHS; 5, apoenzyme inactivated by indomethacin-NHS; 6, 1.5 μM free heme.

Effect of Indomethacin and Indomethacin-NHS on Heme Binding. The effect of indomethacin and indomethacin-NHS on heme reconstitution of apoenzyme was studied. A UV–visible spectrum was recorded of apoenzyme that had been inactivated with 2 equiv of indomethacin or indomethacin-NHS for 30 min and then reconstituted with 1 equiv of hematin (Figure 7). These spectra were compared to the absorption spectrum of holoenzyme at 410 nm. Heme bound to apoenzyme exhibits a Soret maximum at 410 nm with a molar absorptivity of 123 mM^{-1} cm^{-1} (Kulmacz & Lands, 1984). In contrast, aqueous solutions of hematin exhibit an absorbance maximum at 390 nm with an absorptivity at 410 nm of 35 mM^{-1} cm^{-1} (Kulmacz & Lands, 1984). The absorbance of the heme added to indomethacin-NHS-inactivated apoenzyme was weaker, and its maximum was shifted to a lower wavelength than heme added to indomethacin-inactivated apoenzyme. Similar observations were made with apoenzyme treated with indomethacin-IM (data not shown). Indomethacin-NHS-inactivated apoenzyme treated with 180 mM hydroxylamine for 1 h before heme reconstitution exhibited an absorption spectrum similar to that of apoprotein reconstituted with heme.

Heme titrations of indomethacin- and indomethacin-NHS-inactivated apoenzyme also suggested differential heme binding (Figure 8). Addition of hematin to indomethacin-inactivated apoprotein reconstituted the enzyme (with an inflection point at 1 heme/subunit) whereas indomethacin-NHS-inactivated protein produced smaller spectral changes that were presumably due to nonspecific binding of heme. These experiments suggested that indomethacin-IM and indomethacin-NHS prevented heme binding by attaching covalently to the enzyme.

Cleavage of the Indomethacin-NHS-Inactivated Apoenzyme by Trypsin. Trypsin cleaves the 70-kDa PGH synthase protein at Arg^{277} into two fragments of 33 and 38 kDa (Chen et al., 1987). In order to assess whether the modified indomethacin derivatives prevent trypsin cleavage at Arg^{277}, apoenzyme inactivated by 2 equiv of indomethacin or indomethacin-NHS was compared to untreated apoenzyme for its sensitivity to trypsin. In a separate experiment, apoenzyme treated with either indomethacin or indomethacin-NHS was extensively dialyzed before trypsin treatment. The reaction

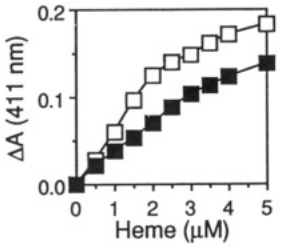

FIGURE 8: Spectrophotometric heme titration of indomethacin- and indomethacin-NHS-inactivated apoPGH synthase. Apoenzyme (200 μg) was treated with 3 equiv of indomethacin or indomethacin-NHS at 0 °C for 3 h in 10 mM Tris-HCl (pH 7.5). Both incubations [2 μM final concentration in 1 mL of 50 mM phosphate (pH 7.5)] were titrated with additions of hematin from a 100 μM stock solution as described in Materials and Methods. The increase in absorbance at 411 nm is plotted for indomethacin (□) or indomethacin-NHS (■) inactivated enzyme.

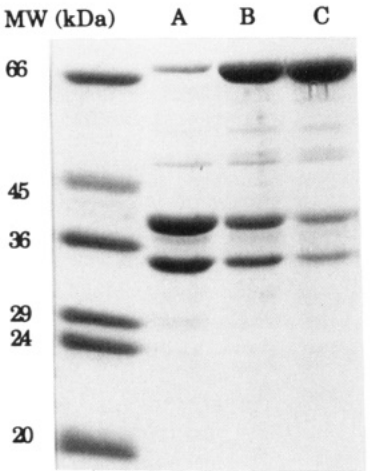

FIGURE 9: Gel electrophoresis of trypsin-cleaved inactivated apoenzyme. Apoenzyme (50 μg) was inactivated by 2 equiv of indomethacin (lane B) or indomethacin-NHS (lane C) for 50 min and was compared to control apoenzyme (lane A) in its ability to be cleaved by trypsin as described in Materials and Methods. A 20-μg aliquot of each sample was loaded on a 10% polyacrylamide gel. The percentage of each band was estimated by densitometry. % 70 kDa remaining: lane A, 11%; lane B, 47%; lane C, 66%.

mixtures were analyzed by polyacrylamide gel electrophoresis, and the percentage of each band was measured by densitometry (Figure 9). Trypsin cleavage was decreased for apoenzyme inactivated with indomethacin and indomethacin-NHS compared to the control. Approximately 89% of the untreated apoenzyme was cleaved compared to 53% and 34% for indomethacin- and indomethacin-NHS-inactivated enzyme, respectively.

Chromatographic Analysis of Labeled Protein. Upon precipitation under nondenaturing conditions of apoPGH synthase inactivated with 2 equiv of [^{14}C]indomethacin-NHS, radioactivity was found associated with the protein. A control performed by treatment of apoenzyme with the same amount of radiolabeled indomethacin revealed almost no radioactivity associated with the protein (results not shown). This suggested that inactivation of apoprotein by indomethacin-NHS was associated with covalent attachment of indomethacin. We attempted to detect the modified protein by reversed-phase HPLC analysis. Apoenzyme was treated with 1 equiv of radiolabeled indomethacin-NHS for 45 min at 0 °C and then the mixture was injected on a Vydac C_4 column eluted with a water–acetonitrile gradient containing 0.1% TFA. Most of the radioactivity eluted in peaks at 6.5 and 8.5 min; very little radioactivity eluted with the protein peak at 21 min (Figure 10). The peaks at 6.5 and 8.5 min corresponded to the retention

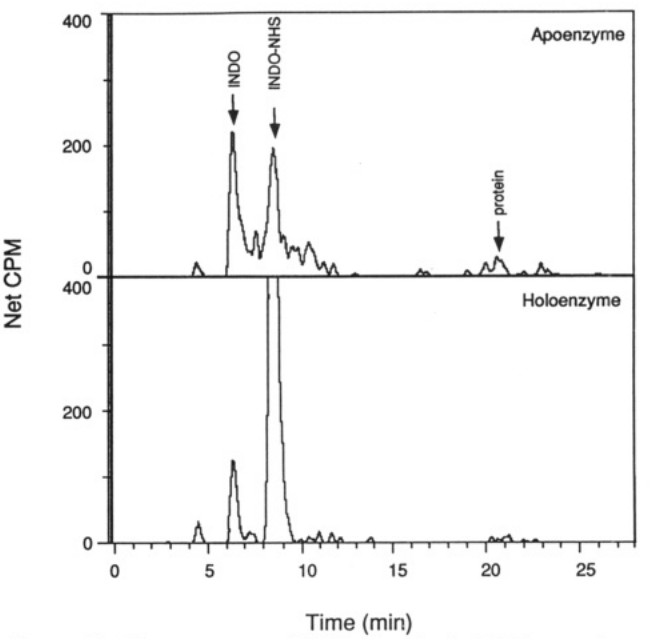

FIGURE 10: Chromatograms of [^{14}C]indomethacin-NHS-treated apo- and holoPGH synthase. Apo- or reconstituted holoenzyme (120 μg) was treated with 1 equiv of radiolabeled indomethacin-NHS for 45 min at 0 °C. An aliquot (60 μg) was injected on a Vydac C_4 column eluted as described in Materials and Methods. The HPLC eluant was monitored with a UV detector (not shown) and a radioactive flow detector. (INDO, indomethacin.)

times of indomethacin and indomethacin-NHS, respectively. Indomethacin-NHS was stable to the HPLC conditions. Its half-life toward hydrolysis was found to be of approximately 145 min in 10 mM Tris-HCl (pH 7.5).

The HPLC profiles of apo- and holoenzyme (60 μg) inactivated by 1 equiv of [^{14}C]indomethacin-NHS for 45 min were compared (Figure 10). The total amount of radioactivity eluting from the column was 4425 cpm for the apoenzyme and 6468 cpm for the holoenzyme. The percentage of radioactivity coeluting with a standard of indomethacin-NHS was 31% for the apoenzyme and 89% for the holoenzyme. The percentage of radioactivity coeluting with indomethacin was 35% for the apoenzyme and 10% for the holoenzyme. Several additional minor peaks were observed with the apoenzyme. Differential hydrolysis of indomethacin-NHS on incubation with apoenzyme and holoenzyme is consistent with attachment of the indomethacin moiety to a protein nucleophile on the apoprotein but not on the holoprotein. Hydrolysis of the indomethacin adduct could occur in the incubation mixture or under the acidic conditions of the HPLC. The fact that no radioactivity coeluted with the protein peak suggests that the covalent indomethacin–protein adduct is less stable than indomethacin-NHS.

Competition with Indomethacin. The foregoing experiments suggested that indomethacin-NHS covalently modifies apoPGH synthase and prevents heme binding to the active site. To determine if the site of covalent attachment is also the indomethacin binding site, the ability of indomethacin and indomethacin-NHS to compete with each other was determined. Two equivalents of indomethacin were reacted with one equivalent of apoPGH synthase for 15 min. The cyclooxygenase activity was inhibited by greater than 83%, but the peroxidase activity was not inhibited. Addition of indomethacin-NHS to indomethacin-treated enzyme led to loss of peroxidase activity at a rate comparable to that of apoenzyme treated with indomethacin-NHS in the absence of indomethacin (Figure 11). Thus, incubation of in-

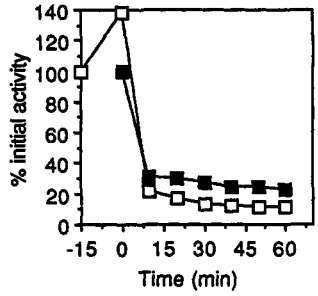

FIGURE 11: Inactivation by indomethacin-NHS of the peroxidase activity of apoPGH synthase and indomethacin-treated apoPGH synthase. ApoPGH synthase (16 μM subunit concentration) was treated with indomethacin (33 μM final concentration) for 15 min at 0 °C. The O_2 uptake assay indicated its cyclooxygenase activity was inhibited by greater than 83%. Indomethacin-NHS was added to a final concentration of 33 μM and incubated at 0 °C for varying times. The time of addition of indomethacin-NHS is indicated as 0 min in the figure. Aliquots were periodically removed and assayed for peroxidase activity. A parallel experiment was performed without indomethacin pretreatment of the apoprotein. (□) With indomethacin pretreatment; (■) without indomethacin pretreatment.

domethacin with apoPGH synthase does not prevent inactivation by subsequent treatment of the protein with indomethacin-NHS.

DISCUSSION

Previous reports from our laboratory and Ruf's laboratory demonstrated that NAI inactivates the cyclooxygenase and peroxidase activities of ovine PGH synthase (Wells & Marnett, 1992; Scherer et al., 1992). The presence of the heme prosthetic group protected the protein from inactivation, and acetylated protein was not reconstituted by heme (Scherer et al., 1992). Thus, it appeared that NAI was reacting with an amino acid residue near the heme binding site and preventing reconstitution of the apoprotein. Since the heme prosthetic group is required for both cyclooxygenase and peroxidase activities, this would account for the inactivation of both of PGH synthase's catalytic activities. Inactivation was reversed by treatment of the acetylated protein with hydroxylamine, which indicated that acetylation of a serine, threonine, or lysine residue could not account for inactivation. Thus, acetylation of PGH synthase by NAI could be distinguished from acetylation by acetylsalicylic acid because the latter selectively acetylates Ser^{530} (Van Der Ouderaa et al., 1980; DeWitt et al., 1990).

Attempts to identify by peptide mapping the residues of PGH synthase acetylated by [*acetyl*-^{3}H]NAI were complicated by the high concentrations of NAI required for inactivation, which led to incorporation of many acetyl groups into the apoprotein (Wells & Marnett, 1992). No differences were detected in the peptide maps derived from apo- and holoenzyme, which suggested that all of the detectable radiolabeled peptides were formed by nonspecific acetylation (Wells & Marnett, 1992). These results also suggested that the acetylated residue responsible for reduced heme binding and enzyme inactivation was unstable and did not survive the conditions employed for peptide mapping. However, these conclusions could only be considered tentative because of the difficulty in detecting one radiolabeled peptide in a complex mixture.

Indomethacin-IM and indomethacin-NHS inactivate both the cyclooxygenase and peroxidase activities of PGH synthase when incubated with apoprotein at a ratio as low as one inhibitor molecule per enzyme subunit. As observed with NAI, the heme prosthetic group protects against inactivation. Inactivated protein binds less heme than untreated protein; the amount of heme binding that does occur is probably due to a hydrophobic site on the protein that is not associated with catalytic activity. Nonspecific binding of heme to PGH synthase has been reported by several other laboratories (Van Der Ouderaa et al., 1979; Roth et al., 1981; Kulmacz & Lands, 1984).

The most straightforward mechanism to explain the inactivation of the catalytic activities of PGH synthase by indomethacin-IM or indomethacin-NHS is modification of a residue on the protein, which prevents binding of the heme prosthetic group. Considering the similarity in the effects of NAI, indomethacin-IM, and indomethacin-NHS, it seems reasonable to suggest they are all modifying the same amino acid residue. However, high concentrations of NAI are required to modify this residue so that modification of other residues occurs simultaneously. This leads to the formation of many radioactively labeled peptides when apoPGH synthase is reacted with [*acetyl*-^{3}H]NAI (Wells & Marnett, 1992). In contrast, the target residue is modified at very low concentrations of indomethacin-IM and indomethacin-NHS, so no other residues are modified.

Reaction of [2-^{14}C]indomethacin-NHS with apoPGH synthase leads to incorporation of [^{14}C] into the protein, but no labeled protein is detectable when it is analyzed by reversed-phase HPLC. The acylated protein appears unstable to the conditions of chromatography. Consistent with this is the detection of higher amounts of the hydrolysis product indomethacin following reaction of [2-^{14}C]indomethacin-NHS with apoPGH synthase compared to holoPGH synthase. Indomethacin-NHS itself is stable to the incubation, workup, and chromatography conditions, so the only way to generate indomethacin from it is to transfer the acyl moiety to a protein residue followed by hydrolysis during chromatography of the acylated protein. It is possible that hydrolysis of acylated enzyme occurs in the reaction mixture prior to HPLC analysis, but earlier experiments indicated that no recovery of cyclooxygenase and peroxidase activities occurred following overnight dialysis against neutral buffers.

The identity of the amino acid residue to which the indomethacin moiety attaches is uncertain. Reversal of inactivation by hydroxylamine rules out serine and lysine residues (Riordan et al., 1965). Reversal of inactivation by incubation at elevated pH's is similar to the behavior of PGH synthase inactivated by NAI, which Scherer et al. attributed to acetyltyrosine (Scherer et al., 1992). However, the instability of acylated PGH synthase to chromatography seems to exclude tyrosine as a potential site for acylation. We find that *N,O*-diacetyltyrosine is recovered quantitatively after its incubation under the conditions used for inactivation of PGH synthase and HPLC analysis (Wells & Marnett, 1992). This suggests the targets for covalent attachment of indomethacin-NHS are histidine or cysteine residues.

The reciprocal relationship between heme binding and indomethacin-NHS inactivation of apoPGH synthase suggests that acylation occurs at the heme binding site or at a region that prevents heme binding by inducing a conformational change in the protein. Indomethacin is known to induce a conformational change in apoPGH synthase as judged by the resistance of indomethacin-treated protein to cleavage at Arg^{277} by trypsin (Kulmacz & Wu, 1989). Indomethacin-NHS also induces resistance to trypsin cleavage. Thus, it is conceivable that this results from the same conformational change induced by indomethacin. However, indomethacin treatment of apoPGH synthase does not prevent binding of

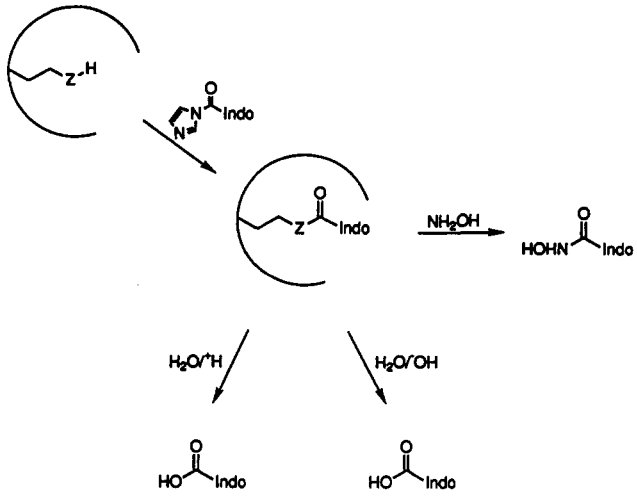

FIGURE 12: Model for inactivation of apoPGH synthase by indomethacin-IM or indomethacin-NHS.

the heme prosthetic group, and holoPGH synthase is sensitive to inhibition by indomethacin. Both observations contrast with the effects of indomethacin-NHS on apo- and holoPGH synthase. Furthermore, indomethacin treatment of apoPGH synthase does not prevent inactivation of its peroxidase activity by subsequent treatment of the protein with indomethacin-NHS. This suggests that indomethacin and indomethacin-NHS bind simultaneously to two different sites on the protein. Indomethacin binds tightly but noncovalently whereas indomethacin-NHS binds covalently.

The simplest explanation for all our results is that indomethacin-NHS covalently binds to an amino acid residue in the heme binding site of the apoprotein (Figure 12). This prevents heme association with the protein and induces a conformational change that renders the protein resistant to trypsin cleavage. Heme reconstitution before treatment with indomethacin-NHS prevents covalent modification of the protein. Potential sites for attachment of indomethacin-NHS include His^{309}, which is believed to be one of the ligands to the heme iron and Cys^{313}, which is only four residues away (Shimokawa & Smith, 1991).

Although the identity of the amino acid target for inactivation of PGH synthase by indomethacin-IM and indomethacin-NHS is uncertain, both indomethacin derivatives are potent and selective modifiers of this protein. Indomethacin-NHS is a particularly useful molecule because it is easily synthesized and stable to handling and incubation at neutral pH. The presence of a carboxylic acid functional group in many NSAID's suggests the strategy of converting this moiety to a reactive acylating derivative could be very useful for selective inactivation, affinity labeling, etc. Such experiments may be very useful for probing the structural differences between the classical PGH synthase studied here and the recently discovered PGH synthase that is 60% identical to it and is regulated by mitogenesis (Xie et al., 1991; Kujubu et al., 1991).

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