Acetylation of Prostaglandin Endoperoxide Synthase by *N*-Acetylimidazole: Comparison to Acetylation by Aspirin†

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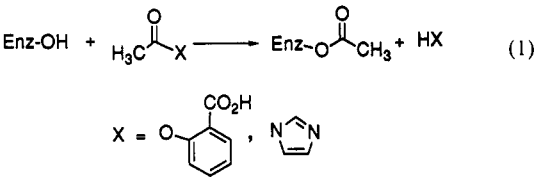
ABSTRACT: Treatment of prostaglandin endoperoxide (PGH) synthase apoprotein with a 100- or 1000-fold excess of *N*-acetylimidazole (NAI) led to time-dependent inactivation of both cyclooxygenase and peroxide activities. Reconstitution of apoprotein with heme prior to incubation with NAI substantially protected the enzyme from inactivation. Pretreatment of the protein with either acetylsalicylic acid (aspirin) or (±)-2-fluoro-α-methyl-4-biphenylacetic acid (flurbiprofen), which inhibit cyclooxygenase activity, did not alter the time course of peroxidase inactivation by NAI. Treatment of NAI-inactivated apoPGH synthase with hydroxylamine led to substantial regeneration of both cyclooxygenase and peroxidase activities. Quantitation of radioactivity following incubation of PGH synthase with [^{3}H-*acetyl*]NAI indicated incorporation of 1.7 ± 0.9 acetyl groups/70-kDa subunit. Cleavage of acetylated protein with trypsin under nondenaturing conditions followed by high-performance liquid chromatography analysis demonstrated that most of the radioactivity was incorporated into the 33-kDa fragment although significant radioactivity was also detectable in the 38-kDa fragment. Chymotryptic peptide mapping of acetylated protein revealed numerous potential sites of acetylation distributed in widely divergent regions of the protein. No apparent differences were observed between the chymotryptic maps of apo- and holoenzyme, suggesting that the adduct responsible for loss of catalytic activity is unstable to the chromatographic conditions. The different biochemical properties of PGH synthase acetylated by NAI or aspirin suggest that a major determinant of the specificity of aspirin for Ser^{530} is binding of the salicylate moiety to this region of the PGH synthase protein.

Prostaglandin endoperoxide (PGH)[1] synthase (EC 1.14.99.1) catalyzes the first two steps of the branch of the arachidonic acid cascade leading to prostaglandins, thromboxane, and prostacyclin (Smith & Marnett, 1991). Its cyclooxygenase activity oxidizes arachidonic acid into PGG_2, and its peroxidase activity reduces PGG_2 into PGH_2 in the presence of a reducing substrate (Hamberg et al., 1974; Nugteren & Hazelhof, 1973). PGH synthase is a homodimer of 70-kDa subunits that binds one heme group per subunit (Van Der Ouderaa et al., 1977; Ruf et al., 1984). Heme is required for both the cyclooxygenase and peroxidase activities.

A distinctive feature of PGH synthase catalysis is the inhibition of its cyclooxygenase activity by aspirin (acetylsalicylic acid) (Vane, 1971). Aspirin transfers its acetyl group to Ser^{530} (Roth & Majerus, 1975; Van Der Ouderaa et al., 1980; Rome et al., 1976)[2] (eq 1), which blocks the approach of arachidonic acid to the substrate binding site (DeWitt et al., 1990). Studies with [^{3}H-*acetyl*]aspirin indicate that Ser^{530} is the only residue on the protein that is acetylated and that the heme prosthetic group dramatically enhances the rate of acetyl transfer (Van Der Ouderaa et al., 1980; Chen & Marnett, 1989; Kulmacz, 1989). One possible explanation for the selective acetylation of Ser^{530} by aspirin is that it possesses the most nucleophilic hydroxyl group in the protein. The enhanced nucleophilicity might result from a heme-induced conformational change that juxtaposes electron-rich residues with Ser^{530} (as in the case of serine proteases). Another explanation for the preferential acetylation of Ser^{530} by aspirin is that it is determined by the specific binding of the salicylate moiety to that region of the protein. This process may be facilitated by the heme prosthetic group.



(1)

If Ser^{530} is the most nucleophilic hydroxyl group in PGH synthase, it should demonstrate enhanced reactivity toward other acetyl-transferring reagents. Therefore, we undertook a study of the reaction of PGH synthase with *N*-acetylimidazole (NAI) (eq 1). NAI acetylates protein residues at rates proportional to their nucleophilicity and accessibility (Riordan et al., 1965). It is commonly used for acetylation of tyrosine hydroxyl groups but will also acetylate cysteine thiol and lysine amino groups under mild conditions (Riordan et al., 1965; Angeles et al., 1989; Kulanthaivel et al., 1989; Wuarin et al., 1989; Cronin & Harbury, 1965; Kaliman et al., 1991; Blumgrund de Satz & Santome, 1981; Argüello & Kaplan, 1990). It does not appear to acetylate serine hydroxyl groups because of their relatively low reactivity at neutral and mildly alkaline pH (Riordan et al., 1965). Acetylation of Ser^{530} of PGH synthase by NAI would be a dramatic demonstration of the nucleophilicity of this residue. However, the results of our experiments demonstrate significant biochemical and

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[1] Abbreviations and trivial names: PGH synthase, prostaglandin endoperoxide synthase; NAI, *N*-acetylimidazole; aspirin, acetylsalicylic acid; flurbiprofen, (±)-2-fluoro-α-methyl-4-biphenylacetic acid; HPLC, high-performance liquid chromatography; TMPD, *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine; TFA, trifluoroacetic acid.

[2] The numbering of amino acids includes the gene signal sequence absent in the mature protein (DeWitt & Smith, 1988).

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functional differences between acetylation of PGH synthase by aspirin and NAI, which indicate that NAI does not acetylate Ser^{530}. A corollary to these findings is that the salicylate moiety plays an important role in the selective acetylation of PGH synthase by aspirin.

MATERIALS AND METHODS

Materials

NAI, imidazole, flurbiprofen, hematin, trypsin (TPCK-treated, type XIII), *N*-acetyltyrosine, *N,O*-diacetyltyrosine, chymotrypsin (TLCK-treated, type VII), and hydroxylamine hydrochloride were purchased from Sigma (St Louis, MO). Arachidonic acid was from Nu Check Prep (Elysian, MN). [^{3}H]Acetic anhydride (50 mCi mmol^{-1}) was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade or better.

PGH synthase was purified from ram seminal vesicles as previously described (Marnett et al., 1984). Apoenzyme was prepared by gel filtration as previously reported (Odenwaller et al., 1990) and 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 20 to 74 μmol of O_2 (mg of protein)$^{-1}$ min^{-1} when assayed in the presence of excess hematin. Holoenzyme was reconstituted by addition to apoenzyme of 1.1 equiv hematin from a 500 μM solution in DMSO.

[^{3}H-*acetyl*]NAI was prepared by reacting equal molar equivalents of imidazole (34 mg, 0.5 mmol), [^{3}H]acetic anhydride (0.5 mmol, 25 mCi), and freshly distilled pyridine (40 μL) in a minimum amount of tetrahydrofuran (170 μL) under nitrogen. NAI precipitated, was separated from the mother liquor, dried under a stream of nitrogen, and stored at −20 °C. The specific activity of the product ranged from 20 to 25 mCi mmol^{-1} in several syntheses. NMR analysis of unlabeled NAI prepared by the same procedure verified the structure of the material and indicated a purity of 97%. The major impurities were the hydrolysis products acetic acid and imidazole.

Methods

Cyclooxygenase Assay. Cyclooxygenase activity was measured at 37 °C with a Gilson Model 5/6H oxygraph 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 by 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, 400 μM H_2O_2, and 500 μM guaiacol or TMPD in 1-mL disposable cuvettes.

Inactivation of Apoenzyme by NAI and Reactivation by Hydroxylamine. A freshly prepared solution of NAI in 10 mM Tris-HCl (pH 7.5) was added in 60- or 1000-fold molar excess to PGH synthase at room temperature; the final enzyme concentration was 10 μM. After inactivation of the enzyme, as judged by the loss of cyclooxygenase and peroxidase activities, hydroxylamine was added from a 2 M solution in 10 mM Tris-HCl (pH 7.5) to a final concentration of 171 mM. Since hydroxylamine interfered with the guaiacol assay at a concentration of 3 mM, TMPD was used as the reducing substrate.

Quantitation of the Number of Residues Acetylated by NAI. Apoenzyme (10 μM) was treated with a 1000-fold excess of [^{3}H-*acetyl*]NAI for 15 or 30 min, and then the excess NAI was removed by overnight dialysis against 10 mM Tris-HCl (pH 7.5). A 100-μL aliquot of the modified enzyme 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 of 52–72% B 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 was monitored at 230 nm. The protein peak was collected at 20–21 min, and its radioactivity was determined by scintillation counting of an aliquot with ScintiVerse scintillation cocktail (Fisher Scientific). The amount of protein injected on the column was determined from a standard curve constructed with known amounts of PGH synthase.

Peptide Mapping of Labeled PGH Synthase. In order to identify the modified residues, 1 mg of apoenzyme or holoenzyme was treated with a 100-fold molar excess [^{3}H-*acetyl*]NAI for 30 min. After dialysis against 10 mM Tris-HCl (pH 7.5) containing 0.1 M $CaCl_2$ to remove excess radioactivity, the enzyme was digested with 2% (w/w) chymotrypsin for 40 h at room temperature, then the peptides were injected on a Vydac C_{18} column (0.46 × 25 cm) using the same solvent system described above. The column was eluted at a flow rate of 1 mL min^{-1} with a 115-min gradient (0–50% B in 75 min, 50–75% B in 25 min, 75–100% B in 15 min). The absorbance was monitored at 230 nm with a Varian 2050 UV detector, and the radioactivity was detected by scintillation counting with a Radiomatic Flo-one β system connected in series.

Amino Acid Sequencing. The peptides of interest were collected from the HPLC and dried on a Savant Speedvac. The residue was dissolved in 0.1% TFA-50% acetonitrile and injected on a Vydac C_{18} column eluted with a gradient of 10–30% solvent B in 60 min. The collected fractions were subjected to amino acid sequencing by automated Edman degradation at the Protein Chemistry Core Facility, Vanderbilt University, Nashville, TN.

RESULTS

Inactivation by NAI. Treatment of apoPGH synthase with NAI led to a rapid loss of cyclooxygenase and peroxidase activities (Figure 1). Both activities decreased at comparable rates that were dependent on the concentration of NAI. The decrease in activity followed an exponential time course with pseudo-first-order rate coefficients for inactivation of 0.0027 and 0.025 min^{-1} at ratios of NAI-to-enzyme of 60 and 1000, respectively. The kinetics of the loss of enzyme activities may be complicated by the decomposition of NAI which occurs concomitant with protein acetylation. NAI hydrolyzes at room temperature with a half-life of 30 min in 10 mM Tris-HCl (pH 7.5),[3] as measured by its UV absorbance (235–255 nm). Reconstitution of apoPGH synthase with 1 equiv of heme substantially protected the protein from inactivation by NAI (Figure 1).

Aspirin acetylation of PGH synthase leads to loss of cyclooxygenase but not peroxidase activity (Miyamoto et al., 1976). In order to test whether the acetylation of Ser^{530} prevented inactivation by NAI, apoenzyme was incubated with a 70-fold excess of aspirin at room temperature until

[3] This pH was used since it is the optimum for the stability of NAI (Riordan et al., 1965).

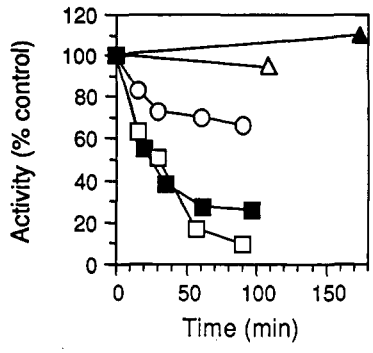

FIGURE 1: Time courses for inactivation of cyclooxygenase and peroxidase activities by NAI. Apo- or holoPGH synthase (10 μM, 180 μg) was incubated with a 1000-fold excess of NAI at room temperature in 10 mM Tris-HCl at pH 7.5. At various time points, 15-μg enzyme aliquots were tested for cyclooxygenase (□) and peroxidase (■) activity of apoenzyme and for cyclooxygenase (○) activity of holoenzyme as described under Materials and Methods. Results are plotted as the percent of the cyclooxygenase (△) and the peroxidase (▲) activities of the control (without NAI) at $t = 0$. The peroxidase was assayed in triplicate for each time point.

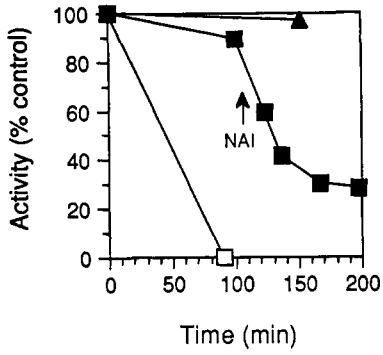

FIGURE 2: Inactivation of the peroxidase activity of aspirin-treated PGH synthase by NAI. ApoPGH synthase (10 μM, 350 μg) was incubated with 0.7 mM aspirin to inactivate its cyclooxygenase activity (□), and then a 1000-fold excess of NAI was added at room temperature at the time indicated by the arrow. At various time points, 15-μg enzyme aliquots were tested for peroxidase activity (■) as described under Materials and Methods. Results are plotted as the percent of the cyclooxygenase and the peroxidase (▲) activities of the control (without NAI) at $t = 0$. The peroxidase activity was assayed in triplicate for each time point.

none of the initial cyclooxygenase activity remained. As expected, there was little loss of peroxidase activity. Addition of a 1000-fold excess of NAI induced a rapid decrease in peroxidase activity, the rate of which was comparable to that observed during the incubation of apoPGH synthase with NAI alone (Figure 2).

Similar experiments were conducted in which apoenzyme was treated with flurbiprofen followed by NAI. Flurbiprofen does not covalently modify the protein but binds to it tightly, leading to a loss of cyclooxygenase but not peroxidase activity. The peroxidase activity of PGH synthase incubated with an 80-fold excess of flurbiprofen decreased rapidly following treatment with a 1000- or a 100-fold excess of NAI (Figure 3). Thus, binding of the enzyme prosthetic group prevented inactivation of PGH synthase by NAI whereas covalent or noncovalent binding of cyclooxygenase inhibitors did not.

Reactivation by Hydroxylamine. As noted by Riordan et al. (1965), acetylation of carboxypeptidase A by NAI can be reversed by a nucleophilic reaction buffer. Similarly, both cyclooxygenase and peroxidase activities were slowly regenerated when apoPGH synthase was incubated with NAI in the buffer used in our enzyme purifications [80 mM Tris-HCl, 300 μM DDC, 0.1% Tween-20 (pH 8)]. However, a

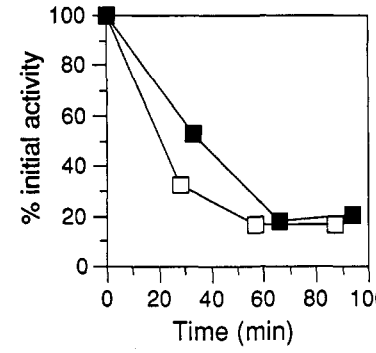

FIGURE 3: Effect of NAI on the peroxidase activity of flurbiprofen-treated PGH synthase. Apoenzyme (22 μM, 250 μg) was pretreated with an 80-fold excess of flurbiprofen to inactivate its cyclooxygenase activity, and then a 1000-fold excess of NAI was added. Aliquots (15 μg) were assayed for peroxidase activity (□). An identical experiment was performed without preincubation with flurbiprofen (■). The peroxidase was assayed in triplicate for each time point.

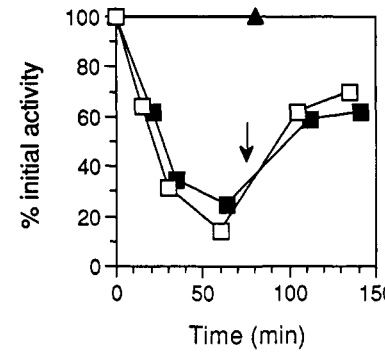

FIGURE 4: Inactivation of cyclooxygenase and peroxidase activities of PGH synthase by NAI and reactivation by hydroxylamine. ApoPGH synthase (14 μM, 340 μg) was inactivated with 14 mM NAI at room temperature in 10 mM Tris-HCl at pH 7.5, and then 171 mM hydroxylamine was added as indicated by the arrow. At various time points, 15 μg of enzyme was removed and tested for cyclooxygenase (□) and peroxidase (■) activity as described under Materials and Methods. Results are plotted as percent of the cyclooxygenase and the peroxidase (▲) activities of the control (without NAI) at $t = 0$. The peroxidase was assayed in triplicate at each time point.

10-mM concentration of Tris-HCl (pH 7.5) did not reverse protein acetylation, and both enzyme activities were still inhibited upon overnight dialysis at 4 °C against this buffer. Thus, covalent modification of the protein is likely.

Hydroxylamine is able to reverse the acylation of tyrosyl and histidyl residues at neutral pH by forming the corresponding hydroxamic acids (Riordan & Vallee, 1971a,b; Wong, 1991). This is usually accompanied by regeneration of enzyme activity (Bünning et al., 1978). Deacetylation of lysine groups by hydroxylamine requires alkaline conditions (Bünning et al., 1978; Means & Feeney, 1971; Argüello & Kaplan, 1990). Both cyclooxygenase and peroxidase activities of NAI-inactivated PGH synthase were restored to about 60% of their initial values upon incubation with 171 mM hydroxylamine in 10 mM Tris-HCl (pH 7.5) for 30 min (Figure 4). However, the aspirin-acetylated enzyme was resistant to hydroxylamine under the same conditions, and no cyclooxygenase activity was recovered. This confirms the fact that different types of residues are being modified by the two different acetylating agents.

Quantitation of Incorporated Acetyl Residues. Aspirin incorporates only one acetyl group per molecule of enzyme (Van Der Ouderaa et al., 1980). The number of amino acid residues modified by treatment of PGH synthase with a 1000-fold molar excess of [^{3}H-*acetyl*]NAI for 15–30 min was

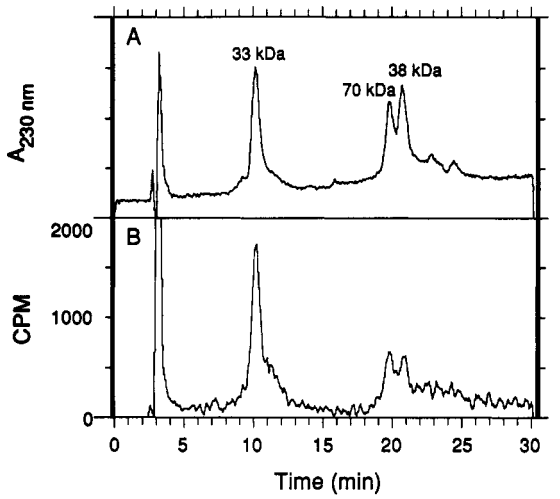

FIGURE 5: Chromatogram of tryptic fragments from [^{3}H-acetyl]-labeled PGH synthase. PGH synthase (120 μg) was treated with 188 μg of [^{3}H-*acetyl*]NAI for 30 min. The modified enzyme was dialyzed overnight as described under Materials and Methods and then incubated with 1.5% (w/w) trypsin for 30 min at room temperature. An aliquot (60 μg) was injected on a Vydac C_4 column eluted as described under Materials and Methods. The fragments were monitored with a UV detector (A) and a radioactive flow detector (B).

Table I: Radioactivity Counts (cpm) in the Protein and Trypsin Cleavage Fragments before and after Treatment by Hydroxylamine[a]

peak	solvent front	33 kDa	70 kDa	38 kDa
before NH_2OH addition (cpm)[b]	9896	8332	4316	3185
after NH_2OH addition (cpm)[c]	27852	4937	1676	2210

[a] Apoenzyme (9 μM) was treated with a 1000-fold excess of [^{3}H-*acetyl*]NAI for 30 min. After extensive dialysis to remove the excess radioactivity, the protein was digested with 1.5% (w/w) trypsin for 30 min. [b] Half of the cleaved protein was injected on a C_4 column as described under Materials and Methods. [c] The other half was treated beforehand with 120 mM hydroxylamine for 15 min. The major peaks were collected as they eluted from the UV detector and were counted in a scintillation counter.

estimated after extensive dialysis of the excess radioactivity, injection of the labeled protein on a C_4 HPLC column eluted with a water–acetonitrile gradient containing 0.1% TFA, and then scintillation counting of the total radioactivity present in the protein peak. The amount of protein was estimated from a standard curve established by HPLC analysis of known quantities of PGH synthase. NAI appeared to modify 1.7 ± 0.9 amino acids per molecule of enzyme.

Trypsin Cleavage of NAI-Labeled Enzyme. In order to evaluate the distribution of radioactivity in the tryptic fragments of PGH synthase and to estimate the extent of deacetylation after hydroxylamine treatment, apoenzyme (9 μM) was modified with a 1000-fold excess of [^{3}H-*acetyl*]-NAI for 30 min and then digested with 1.5% (w/w) trypsin for 30 min. Trypsin cleaves the 70-kDa protein at Arg^{277} into two fragments of 33 and 38 kDa; the serine residue modified by aspirin is located in the 38-kDa fragment (Chen et al., 1987). Half of the digested enzyme was directly injected on a C_4 HPLC column as described above, and the other half was treated with 120 mM hydroxylamine at pH 7.5 for 15 min before HPLC analysis. The radioactivity in the 33-, 38-, and 70-kDa proteins was monitored in each case (Figure 5 and Table I). Although all the peaks were labeled, the peak corresponding to the 33-kDa protein contained most of the radioactivity. Treatment by hydroxylamine decreased the amount of label incorporation by about half for each peak,

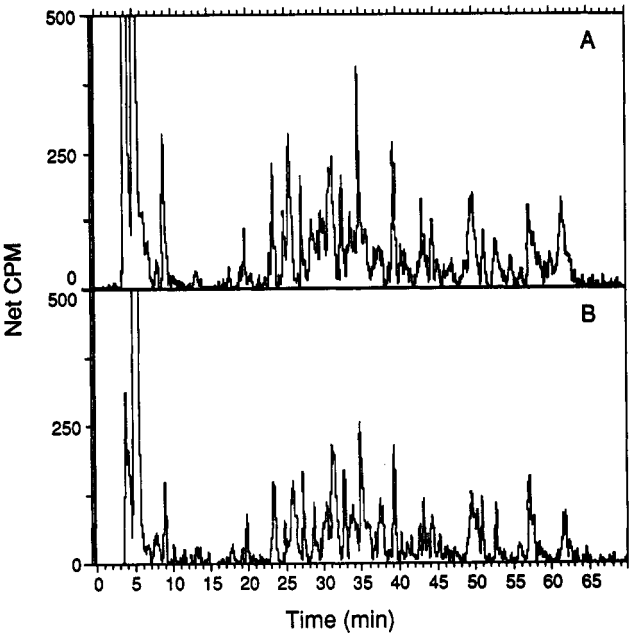

FIGURE 6: Peptide mapping of NAI-modified PGH synthase. One milligram of apoenzyme (A) or holoenzyme (B) was treated with a 100-fold excess of [^{3}H-*acetyl*]NAI for 30 min. After dialysis, the protein was digested with 2% chymotrypsin for 40 h. The peptides were injected on a Vydac C_{18} column eluted as described under Materials and Methods. The HPLC eluant was monitored with a UV detector and a radioactive flow detector. Only the radioactive traces are presented. The profiles are typical of those obtained from three separate mapping experiments.

and most of the radioactivity eluted with the solvent front, probably as acetylhydroxamic acid.

Peptide Mapping of [^{3}H-acetyl]NAI-Modified PGH Synthase. In an attempt to identify regions of the protein where acetylation occurred, NAI-modified PGH synthase was submitted to peptide mapping using chymotryptic digestion and reverse-phase HPLC analysis. After treatment of apoenzyme with a 1000-fold excess [^{3}H-*acetyl*]NAI for 30 min followed by a 24-h digestion by 2% chymotrypsin, HPLC analysis revealed many radiolabeled peptides. Therefore, acetylation was carried out with a 100-fold excess of radiolabeled NAI for 30 min. Chymotrypsin digestion was conducted under nondenaturing conditions in the absence of thiol reductants and cysteine acetylating agents to minimize hydrolysis or decomposition of acetylated residues prior to HPLC analysis.

A typical peptide map of depicted in Figure 6. Major radioactive peaks were observed between 20 and 35 min. Additional radioactive peaks eluted as the percent of organic solvent increased, but these peaks appeared to represent incompletely digested peptides. The major early eluting radioactive peaks were collected and rechromatographed using a shallower gradient. Radioactive peaks from the second chromatography were subjected to amino acid sequencing. The positions in the PGH synthase sequence of the peptides identified were 231–234 (Gly-His-Ile-Tyr), 415–417 (Val-Asp-Tyr), 76–77 (Thr-Trp), 108–112 (Ile-Arg-Asp-Thr-Leu), 202–205 (Ala-Gln-His-Phe), and 225–230 (Gly-His-Gly-Val-Asp-Leu). Precise definition of the site of attachment of the acetyl moiety in the isolated peptides was not possible because of the instability of the acetylated amino acids under the conditions of the Edman degradation. The detection of peptides in which a histidine residue was the only potential site of acetylation was surprising because it was anticipated that *N*-acetylhistidine residues in small peptides would hydrolyze during the multiple chromatographic steps that

preceded amino acid sequencing. Each of the peptides was repurified before sequence analysis and chromatographed as a single peak on HPLC, but it is possible that the radioactivity in the final peak submitted for sequencing was due to a small amount of a contaminating radiolabeled peptide.

No significant differences were observed between the chymotryptic maps of apoPGH synthase or holoPGH synthase treated with radiolabeled NAI for 30 min (Figure 6). Since the heme prosthetic group protected PGH synthase from inactivation by NAI (Figure 1), the similarity of the peptide maps suggests that none of the peptides detected in the chymotryptic maps of acetylated apoprotein or holoprotein contain the acetylated amino acid that is responsible for inactivation. This implies that the acetylated residue responsible for inactivation is unstable to the conditions of the proteolytic digestion and chromatography. A standard of *N,O*-diacetyltyrosine underwent less than 1% O-deacetylation to *N*-acetyltyrosine when it was exposed to the conditions used for peptide mapping. However, *N,O*-diacetyltyrosine was quantitatively converted to *N*-acetyltyrosine after incubation with 230 mM hydroxylamine for 30 min. This finding suggests that the NAI-acetylated residue that leads to inactivation of PGH synthase is not *O*-acetyltyrosine.

DISCUSSION

The results of the present experiments demonstrate that significant differences exist in the ability of NAI and aspirin to acetylate PGH synthase. (1) Treatment with NAI leads to loss of both cyclooxygenase and peroxidase activities whereas only the cyclooxygenase activity is abolished by aspirin treatment. (2) Inactivation by NAI is inhibited by the presence of the heme prosthetic group whereas inactivation by aspirin is enhanced by the heme group. (3) Acetylation and inactivation of both cyclooxygenase and peroxidase activities by NAI is reversed by hydroxylamine whereas acetylation and inactivation by aspirin are not. (4) Tryptic cleavage of PGH synthase acetylated by NAI reveals that most of the acetyl groups are in the 33-kDa fragment; by contrast, aspirin only introduces one acetyl group in the 38-kDa fragment. (5) NAI introduces acetyl groups at numerous and different types of residues throughout the protein whereas aspirin acetylates only Ser^{530}.

Previous studies have shown that the heme prosthetic group accelerates the rate of aspirin acetylation of Ser^{530} at least 100-fold (Chen & Marnett, 1989; Kulmacz, 1989). The ability of heme to protect PGH synthase from inactivation by NAI implies that NAI does not acetylate Ser^{530} in the holoenzyme. This conclusion is also true for NAI acetylation of the apoenzyme. Cyclooxygenase and peroxidase activities are restored to the same extent by treatment of the acetylated protein with hydroxylamine (Figure 4) (Scherer et al., 1992) whereas the cyclooxygenase activity of aspirin-acetylated PGH synthase is not restored by hydroxylamine treatment. The fact that NAI does not acetylate Ser^{530} in either apo- or holoPGH synthase indicates that this hydroxyl group is not the most nucleophilic site in the protein.

The possibility that Ser^{530} is sterically inaccessible to NAI in both the apo- and holoenzyme seems unlikely; molecular modeling indicates that the imidazole group of NAI is actually smaller than the salicylate group of aspirin.[4] Thus, the surface of the protein where aspirin associates with PGH synthase prior to acetyl transfer should also be accessible to NAI. This implies that electrostatic, hydrogen-bonding, or hydrophobic effects are the major determinants of aspirin binding to this region of PGH synthase. Salicylate and certain analogs prevent acetylation of PGH synthase by aspirin at concentrations well below those at which they inhibit cyclooxygenase activity directly (Cerletti et al., 1981, 1983; Humes et al., 1981; Rotilio et al., 1984). Salicylic acid, methyl salicylate, and, to a much lesser extent, 6-hydroxysalicylic acid inhibit acetylation of platelet cyclooxygenase by aspirin, but the 3- and 4-hydroxy isomers of salicylate as well as phenol and benzoic acid do not (Rotilio et al., 1984). Interestingly, all of these compounds inhibit cyclooxygenase activity at higher concentrations (Rotilio et al., 1984). Thus, there appears to be some specificity in the molecular determinants of aspirin association with the region of PGH synthase containing Ser^{530} which are different than those responsible for competitive inhibition of the enzyme by related salicylates.

Site-directed mutagenesis experiments indicate that Ser^{530} is not essential for catalytic activity; the $Ser^{530} \rightarrow Ala^{530}$ mutant of ovine PGH synthase has normal cyclooxygenase activity (DeWitt et al., 1990). However, increasing the steric bulk at this position reduces cyclooxygenase activity by increasing the K_m for substrate binding (DeWitt et al., 1990). Whether Ser^{530} is located in the substrate binding site or in a position to control access to it is uncertain. Regardless of the precise location of Ser^{530}, the present experiments suggest there is a salicylate binding site adjacent to it. Heme enhancement of aspirin acetylation may result from conformationally-induced juxtaposition of these structural elements.

The identity of the functional group acetylated by NAI that leads to inactivation of apoPGH synthase is uncertain. A considerable excess of NAI is required for complete inactivation, and this results in the introduction of several acetyl groups into the protein. Although the number of acetyl groups introduced appears to be approximately two as judged by incorporation of radioactivity from [^{3}H]NAI, the peptide map in Figure 6 reveals more than two acetylated peptides. The identity of the labeled amino acids could not be unequivocally established by sequence analysis, but it appears that several functional groups located in different regions of the protein are acetylated. Interestingly, when holoPGH synthase was acetylated by [^{3}H]NAI, very similar peptide maps were observed. Although we cannot absolutely exclude the possibility that a differentially labeled peptide is obscured by another radiolabeled peptide peak, it seems likely that none of the acetylated peptides detected by mapping contain the amino acid responsible for enzyme inactivation by NAI. This suggests that the critical acetylated peptide is unstable to the conditions of our workup and peptide mapping. If so, the target for acetylation that leads to inactivation may not be a tyrosine residue because the *O*-acetyl group of *N,O*-acetyltyrosine is stable to the conditions employed for peptide mapping.

Although most of the acetylation of PGH synthase by NAI appears nonspecific, inactivation probably results from acetylation in the vicinity of the active site. This is implied by the observation that heme protects against inactivation. Scherer et al. (1992) have recently found that acetylation of PGH synthase by NAI prevents binding of heme to the active site of the apoprotein. Inhibition of heme binding is reversed by addition of hydroxylamine to acetylated protein (Scherer et al., 1992). This parallels the reversal of NAI inactivation of enzyme activity by hydroxylamine and implies that acetylation of the same amino acid residue leads to inhibition of prosthetic group binding and loss of enzyme activity. Scherer et al. (1992) attribute the loss of enzyme activity to acetylation of

[4] L. J. Marnett, unpublished result.

a tyrosine residue because activity is regenerated spontaneously at elevated pHs. However, acetylhistidine and acetylcysteine residues are unstable to hydroxylamine treatment at neutral pH, so histidine or cysteine acetylation must also be considered as possibilities for inactivation (Riordan & Vallee, 1972a). The possible importance of histidine residues in PGH synthase activity was explored by Zhang et al. (1992) using diethylpyrocarbonate as a reagent for covalent modification. Diethylpyrocarbonate inactivates PGH synthase, and Zhang et al. proposed this is due to histidine acylation because of its pH dependence (Zhang et al., 1992). However, hydroxylamine did not regenerate enzyme activity even though it did cause partial regeneration of histidine residues. The reason that hydroxylamine reacted with some acylhistidine residues but not others was not established. Nevertheless, this finding suggests that NAI and diethylpyrocarbonate inactivate PGH synthase by derivatization of different amino acids.

In summary, treatment of PGH synthase with NAI leads to acetylation of different residues that are widely distributed over the protein. Loss of catalytic activity appears to result from acetylation of one or more residues near the active site of the protein. This acetylated residue(s) is stable to dialysis but appears unstable to treatment with hydroxylamine and to the conditions of peptide mapping. Although NAI reacts with several nucleophilic sites in PGH synthase, it does not react with the hydroxyl group of Ser^{530} in either the apoprotein or the holoprotein, which implies that this residue is not the most nucleophilic residue in the protein. Thus, the exquisite sensitivity of Ser^{530} to acetylation by aspirin appears to be determined mainly by specific binding of its salicylate moiety to the region of the PGH synthase protein adjacent to this hydroxyl group.

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