Bromoacetamido Analogs of Indomethacin and Mefenamic Acid as Affinity-Labeling Agents and Mechanistic Probes for Prostaglandin H_2 Synthase[†,‡]

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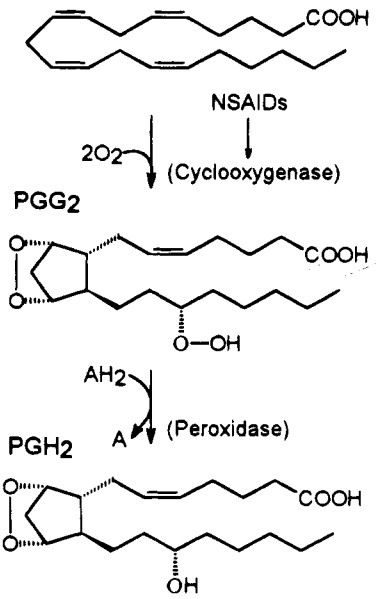
ABSTRACT: Affinity-labeling agents, 1-[4-(bromoacetamido)benzyl]-5-methoxy-2-methylindole-3-acetic acid (**I**) and 4-(bromoacetamido)-*N*-(2,3-dimethylphenyl)anthranilic acid (**II**), were synthesized on the basis of their respective nonsteroidal anti-inflammatory drugs (NSAIDs), indomethacin and mefenamic acid [Askonas & Penning (1991) *Biochemistry 30*, 11553−11560]. Compounds **I** and **II** are now shown to inhibit homogeneous ram seminal vesicle prostaglandin H_2 (PGH_2) synthase by two kinetically distinct complexes. They are competitive inhibitors versus arachidonic acid via the formation of high-affinity E·I complexes, and they cause time-dependent inactivation of the holoenzyme via low-affinity E·I complexes. Compounds **I** and **II**, unlike classical NSAIDs, were found to inactivate both the cyclooxygenase and peroxidase reactions of the synthase in a parallel manner. Inactivation was accompanied by the incorporation of 2 mol of either radiolabeled **I** or **II** per synthase monomer. The covalent bonds that result were stable to boiling in SDS, indicating that **I** and **II** offer alternatives to aspirin in locating NSAID binding sites. Incubation of aspirin-treated PGH_2 synthase with radiolabeled **I** reduced the stoichiometry of incorporation to 1.0, suggesting that one of the sites modified corresponds to the cyclooxygenase site. By saturating the cyclooxygenase site with mefenamic acid, **I** and **II** only abolished the peroxidase activity of the enzyme, suggesting that the second site of modification corresponds to the peroxidase site. When PGH_2 synthase was incubated with mefenamic acid and **I** or **II**, only the peroxidase activity was inactivated. Subsequent removal of all drugs by dialysis gave a preparation of PGH_2 synthase that could perform the cyclooxygenase reaction, but lacked the ability to cleave ethyl hydroperoxide to ethanol and water. The ability to chemically prepare PGH_2 synthase devoid of peroxidase activity questions mechanisms of PGH_2 synthase catalysis that have an obligatory requirement for the peroxidase reaction.

Prostaglandin H_2 (PGH_2)[1] synthase (EC 1.14.99.1) catalyzes both the bis-dioxygenation of arachidonic acid to yield PGG_2 (cyclooxygenase reaction) and the peroxidative cleavage of PGG_2 to yield PGH_2 (peroxidase reaction), as described by Hamburg and Samuelsson (1967, 1973) (Scheme 1). PGH_2 synthase (M_r 72 000) functions as a homodimer and is a target for nonsteroidal anti-inflammatory drugs (NSAIDs; Vane, 1971; Vane et al., 1974; Humes et al., 1981; Vane & Botting, 1987). These drugs inhibit only the cyclooxygenase reaction, block the formation of PGG_2, and prevent the formation of the primary prostaglandins that mediate symptoms of inflammation. NSAIDs in therapeutic use fall into at least three categories with respect to PGH_2 synthase. First, acetyl salicylate (aspirin) acts as an irreversible inhibitor and acetylates Ser 530 (van der Ouderaa et

Scheme 1: Reaction Catalyzed by PGH_2 Synthase[a]



[a] AH_2 is the reducing cosubstrate; the site of NSAID inhibition is indicated.

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[1] Abbreviations: PGH_2, prostaglandin H_2 synthase (EC 1.14.99.1); PGG_2, prostaglandin G_2; NSAID, nonsteroidal anti-inflammatory drug; indomethacin, 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid; 4′-BrAc-indomethacin, 1-[4-(bromoacetamido)benzyl]-5-methoxy-2-methylindole-3-acetic acid; mefenamic acid, *N*-(2,3-xylyl)anthranilic acid; 4′-BrAc-mefenamic acid, 4-(bromoacetamido)-*N*-(2,3-dimethylphenyl)anthranilic acid.

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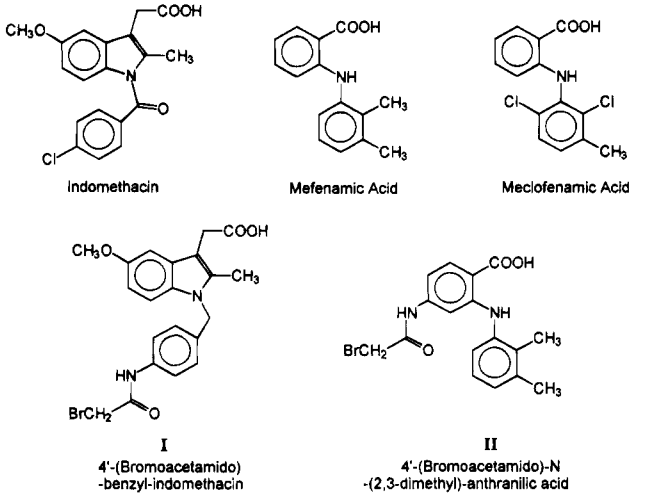
al., 1980). Second, drugs that contain an aryl halide and a carboxylic acid functionality (e.g., indomethacin and meclofenamic acid) are competitive inhibitors against arachidonic acid, but also cause the time-dependent inactivation of free enzyme without resultant covalent modification (Rome & Lands, 1975). Third, the remaining NSAIDs act as pure competitive inhibitors of arachidonic acid (e.g., mefenamic acid).

Although it is known that NSAIDs mediate their actions through one or more of these mechanisms, the topography of the NSAID binding site(s) requires further elucidation. Original models of the NSAID binding site came from studies in which the crystal structure of indomethacin was superimposed onto the flexible structure of arachidonic acid (Appleton & Brown, 1979). Another model was proposed in which the binding sites for NSAIDs and arachidonic acid did not overlap (Kulmacz, 1989; Kulmacz & Wu, 1989). Recently, the X-ray crystal structure of PGH_2 synthase complexed to (*S*)-flurbiprofen has been solved. The structure indicated that the drug binds stereospecifically in a long hydrophobic channel and that Arg 120 acts as a counterion for the carboxylic acid group of the drug (Picot et al., 1994). However, the structure of this enzyme−drug complex does not provide a model in which all NSAIDs can be readily accommodated (D. Picot, P. J. Loll, and R. M. Garavito, personal communication).

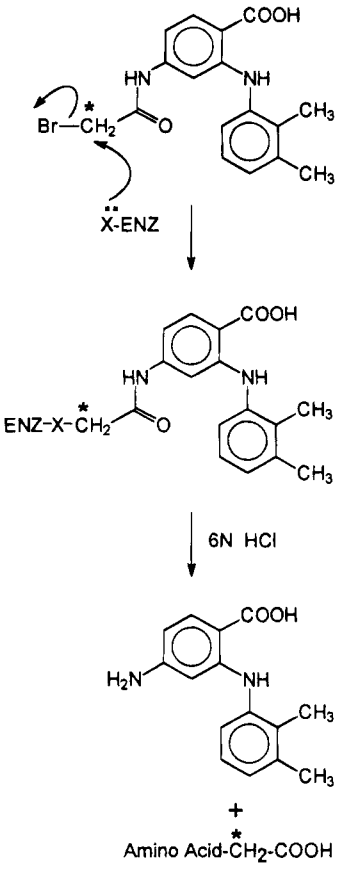
Affinity labeling offers a complementary approach by which NSAID binding sites may be mapped. Recently, acetylating derivatives of indomethacin (indomethacin imidazole and indomethacin *N*-hydroxysuccinimide) were synthesized and found to be potent inactivators of PGH_2 synthase that selectively and covalently modified only the apoenzyme (Wells & Marnett, 1993). These indomethacin derivatives were shown to prevent the binding of heme to the active site. Since NSAIDs inhibit the holoenzyme, there is some question regarding the selectivity of these acetylating agents for the drug binding site. Furthermore, the resultant covalent linkages were unstable, thereby making the isolation and sequencing of radiolabeled peptides untenable.

In an attempt to map the topography of the NSAID binding sites of PGH_2 synthase, we have synthesized affinity-labeling analogs of indomethacin [1-[4-(bromoacetamido)benzyl]-5-methoxy-2-methylindole-3-acetic acid (**I**)] and of mefenamic acid [4′-(bromoacetamido)-*N*-(2,3-dimethylphenyl)anthranilic acid (**II**)] (Askonas & Penning, 1991) (Scheme 2). In each case, the bromoacetamido group is the affinity-labeling tag and nucleophilic attack will yield carboxymethylated PGH_2 synthase (Scheme 3). In this study, the interactions of holo-PGH_2 synthase with the affinity-labeling agents, **I** and **II**, indomethacin, meclofenamic acid, and mefenamic acid were compared. We provide evidence that **I** and **II** (a) have unique pharmacology since, unlike classical NSAIDs, they abolish both the cyclooxygenase and peroxidase activities, (b) label two sites on holo-PGH_2 synthase, which may correspond to the cyclooxygenase and peroxidase sites, and (c) provide a chemical method for obtaining PGH_2 synthase devoid of peroxidase activity. The latter finding has implications for the catalytic mechanism of PGH_2 synthase.

Scheme 2: Nonsteroidal Anti-inflammatory Drugs and Their Analogs



Scheme 3: Affinity Labeling of PGH_2 Synthase with the Bromoacetamido NSAID Analogs[a]



[a] * = ^{14}C and Enz-X refers to a nucleophilic amino acid in the enzyme.

EXPERIMENTAL PROCEDURES

Materials. TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine), arachidonic acid, indomethacin, meclofenamic acid, mefenamic acid, hematin, and alcohol dehydrogenase (baker's yeast) were purchased from Sigma (St. Louis, MO). H_2O_2 (30%, v/v) was purchased from Aldrich Chemical Co. (Milwaukee, WI). EtOOH (ethyl hydroperoxide) was obtained as a 5% solution from Polysciences Inc. (Warrington, PA). NAD^+ (β-nicotinamide adenine dinucleotide, grade II) was purchased from Boehringer Mannheim (Indianapolis, IN). Reagents for the Bradford assay were obtained from Bio-Rad (Hercules, CA). Solvable and Ecolite were obtained from NEN Research Products (Boston, MA).

Enzyme. Prostaglandin H_2 synthase was solubilized (in 0.1% Tween 20 buffer) from ram seminal vesicle microsomes and purified to homogeneity (Marnett et al., 1984). SDS−PAGE of the solubilized preparation showed one band of 72 000, which corresponds to the molecular weight assigned to PGH_2 synthase. Two-dimensional SDS−PAGE further established the purity of the preparation, yielding a single band with pI = 6.5.

Cyclooxygenase Assay. The bis-dioxygenation of arachidonic acid to yield prostaglandin G_2 (PGG_2) was followed by measuring oxygen consumption using a computerized Clark-style oxygen microelectrode (Instech, Plymouth Meeting, PA). The standard assay chamber (600 μL) contained 100 mM Tris-HCl buffer (pH 8.0), 1 mM phenol, 100 μM hematin, and 100 μM arachidonic acid. The reactions were initiated by the addition of PGH_2 synthase. By using this procedure, a specific activity of 21−24 μmol of oxygen consumed/min/mg of enzyme was observed.

Peroxidase Activity: Method I. The two-electron reduction of H_2O_2 using TMPD (*N,N,N′,N′*-tetramethyl-1,4-phenylenediamine) as the reducing cosubstrate was measured spectrophotometrically (Kulmacz & Lands, 1987; Kulmacz, 1989). The reaction system (1.0 mL) contained 35 μg of PGH_2 synthase, 100 mM Tris-HCl buffer (pH 8.0), 1 mM phenol, 100 μM hematin, 80 μM TMPD, and 60 μM H_2O_2 (30% v/v). The reaction was initiated by the addition of TMPD. The formation of *N,N,N′,N′*-tetramethyl-1,4-phenylenediimine (E_{611} = 13 500 M^{-1} cm^{-1}) was complete within 60 s. By using this procedure, a specific activity of 10−12 μmol of TMPD reduced/min/mg of enzyme was observed.

Peroxidase Activity: Method II. The peroxidative cleavage of ethyl hydroperoxide to ethanol and water was measured by coupling ethanol formation to alcohol dehydrogenase and measuring the reduction of NAD^+ to NADH at 340 nm (E_{340} = 6270 M^{-1} cm^{-1}). The reaction system (1.0 mL) contained 50 mM glycine buffer (pH 8.5), 100 μM hematin, 200 μM EtOOH, 2.3 mM NAD^+, and 0.125 unit of alcohol dehydrogenase. The reaction was initiated by the addition of PGH_2 synthase. The assay was validated by showing that the formation of ethanol was linear with respect to time and enzyme concentration. When these concentrations of EtOOH and NAD^+ were incubated with alcohol dehydrogenase, the background rate of formation of NADH was identical to that observed with NAD^+ and alcohol dehydrogenase alone. This indicated that contaminating EtOH did not contribute to the initial velocities observed with PGH_2 synthase. The concentration of EtOOH used was lower than its K_m for PGH_2 synthase [K_m = 0.39 mM (Kulmacz et al., 1990)] to ensure that background rates due to contaminating EtOH were insignificant. By using this procedure, a specific activity of 2 μmol of EtOOH cleaved/min/mg of enzyme was observed.

Radiolabeling **I** *and* **II**. Radiolabeled **I** and **II** were synthesized using either [^{14}C]$BrCH_2$*COOH or its anhydride to couple to the free amine (Askonas & Penning, 1991) and were purified by RP-HPLC to specific radioactivities of 2.24 × 10^4 cpm/nmol for **I** and 1.95 × 10^4 cpm/nmol for **II**. Briefly, radiolabeled **I** or **II** (100 nmol) was resuspended in 600 μL of 40% methanol/H_2O for purification on a C-18 μ-Bondapak column (Waters, Milford, MA) linked to a Beckman System Gold Model 125/166 high-performance liquid chromatograph. Radiolabeled **I** and **II** were eluted with a gradient of 40−70% methanol/H_2O over 30 min, with a flow rate of 0.5 mL/min. The effluent was monitored at 226 nm, and a single major peak, which corresponded to the appropriate synthetic standard, was collected. The specific radioactivities of **I** and **II** were determined using their respective molar extinction coefficients (Askonas & Penning, 1991).

Competitive Inhibition of PGH_2 Synthase by NSAIDs, **I**, *and* **II**. The reversible inhibition of either cyclooxygenase or peroxidase was determined in assays containing either arachidonic acid or TMPD plus H_2O_2 as substrates, respectively, while the drug (indomethacin, meclofenamic acid, mefenamic acid, **I**, and **II**) concentration was varied. Five different substrate concentrations were used for each drug. All assays were performed in triplicate. The drugs were dissolved in DMSO, and the final concentration of organic solvent was 4%. DMSO had a minimal effect on the initial velocities. Initial velocity data were fit to the appropriate rate equation for COMP, UNCOMP, or NONCOMP with the computer programs described by Cleland (1977, 1979). Patterns of inhibition and kinetic constants reported are those judged to be the best fit according to the criteria of Cleland (1979).

Inactivation of PGH_2 Synthase by Indomethacin, Meclofenamic Acid, **I**, *and* **II**. PGH_2 synthase (40.0 μM) was incubated at 25 °C in 100 mM Tris-HCl buffer (pH 8.0), 1 mM phenol, 100 μM hematin, and 5% DMSO (v/v) with increasing concentrations of drug. Time courses were initiated by the addition of the drug. Aliquots were removed over time and diluted 75-fold into the cyclooxygenase or peroxidase assay systems (method I), and the amount of enzyme activity remaining was determined. Semilogarithmic plots of the percent initial enzyme activity versus time were constructed. By drawing tangents to the initial portion of each progress curve, the pseudo-first-order rate constants (k_{app}) were obtained. These data were then analyzed by the method of Kitz and Wilson (1962), in which $1/k_{app}$ is plotted against 1/[I]. This plot yields the K_i values for the E·I complex and half-lives for the enzyme at saturation by each inhibitor.

Incorporation of ^{14}C-Radiolabeled **I** *and* **II** *into PGH_2 Synthase.* PGH_2 synthase (40.0 μM) was incubated in 100 mM Tris-HCl buffer (pH 8.0), 1 mM phenol, 100 μM hematin, and 5% DMSO (v/v) with either 3.0 mM ^{14}C-radiolabeled **I** or 2.0 mM ^{14}C-radiolabeled **II** at 25 °C for 10 h in 80 μL. Aliquots were removed over time and diluted 75-fold into the cyclooxygenase or peroxidase assays (method I), and the amount of enzyme activity remaining was determined. Protein concentrations were redetermined by the method of Bradford (1976), the samples were boiled in SDS and separated by SDS−PAGE to remove excess label, and the gels were stained with Coomassie Blue. Slices containing PGH_2 synthase were excised from the gels, incubated in Solvable (NEN), and counted after the addition of Ecolite (NEN). The stoichiometry of incorporation into PGH_2 synthase was calculated by comparing the amount of protein loaded onto the gel with the radioactivity recovered using the specific activities of ^{14}C-radiolabeled **I** and **II** as conversion factors.

Preparation of PGH_2 Synthase Devoid of Peroxidase Activity. PGH_2 synthase (40.0 μM) was incubated in 100 mM Tris-HCl buffer (pH 8.0), 1 mM phenol, 100 μM hematin, and 5% DMSO (v/v) with either 2.7 mM **I** or 4.5 mM **II** in the presence of 100−150 μM mefenamic acid (to protect the cyclooxygenase activity) in 100 μL at 25 °C for

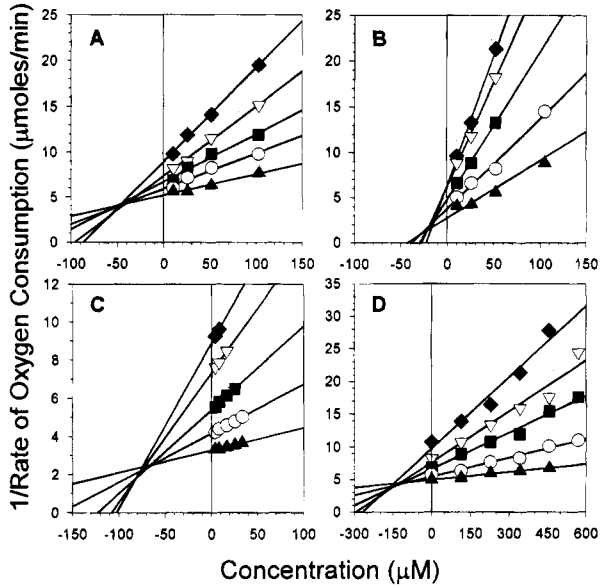


FIGURE 1: Reversible inhibition of the cyclooxygenase activity of PGH_2 synthase by indomethacin (A), mefenamic acid (B), 4-BrAc-indomethacin (**I**) (C), and 4-BrAc-mefenamic acid (**II**) (D). By using the standard cyclooxygenase assay, the concentrations of indomethacin, mefenamic acid, **I**, and **II** were varied against arachidonic acid [20 μM (◆), 25 μM (▽), 33.3 μM (■), 50 μM (○), and 100 μM (▲)]. The data are presented as Dixon plots. The resulting patterns of inhibition were fit to the COMP, UNCOMP, and NONCOMP programs of Cleland (1979), which indicate that indomethacin, mefenamic acid, **I**, and **II** are competitive inhibitors against arachidonic acid.

24 h. Aliquots were removed over time and diluted 75-fold into the cyclooxygenase or peroxidase assays, respectively, and the amount of enzyme activity remaining was determined. In some experiments, all drug was removed by dialysis, and the resultant enzyme displayed 70% of the cyclooxygenase activity of untreated enzyme but did not display any peroxidase activity using either TMPD plus H_2O_2 or ethyl hydroperoxide as substrates.

RESULTS

Competitive Inhibition of the Cyclooxygenase Activity of PGH_2 Synthase by **I** *and* **II**. To determine whether the affinity-labeling agents **I** and **II** can act as competitive inhibitors of arachidonic acid, initial velocity studies were performed. As controls, indomethacin, meclofenamic acid, and mefenamic acid were also examined as reversible enzyme inhibitors. It was found that **I**, **II**, and the three NSAIDs were all competitive inhibitors against arachidonic acid, suggesting that these ligands are mutually exclusive. Representative kinetic data and K_i (inhibition) values for these compounds are given (see Figure 1A–D and Table 1).

Time- and Concentration-Dependent Inactivation of the Cyclooxygenase Activity of PGH_2 Synthase. To assess whether the affinity-labeling agents **I** and **II** can irreversibly inactivate the cyclooxygenase activity of PGH_2 synthase, the holoenzyme was preincubated with these agents and the amount of cyclooxygenase activity remaining over time was determined. As controls, indomethacin, meclofenamic acid, and mefenamic acid were also examined as irreversible enzyme inhibitors. Treatment of the holoenzyme with indomethacin, meclofenamic acid, **I**, and **II** caused time- and concentration-dependent inactivation of the cyclooxygenase activity of PGH_2 synthase (see Figure 2A–C for representative data). For each compound, the progress curves for inactivation appeared to follow pseudo-first-order kinetics. The K_i (inactivation) values for the E·I complex and the half-lives for the enzyme at saturation are given in Table 1.

Table 1: Kinetic Constants for the Inhibition and Inactivation of PGH_2 Synthase

		enzyme inactivation	
compound	enzyme inhibition K_i(inhibition)[a] (μM)	K_i(inactivation) (μM)	$t_{1/2}$[b]
indomethacin	55	7.2	1.4 s
meclofenamic acid	52	4.5	5.6 s
mefenamic acid	13.6		
I	79	930	5.3 h
II	154	2400	3.05 h

[a] K_i for competitive inhibition versus arachidonic acid. [b] $t_{1/2}$ is the time required to inactivate half of the enzyme at saturation.

Both indomethacin and its bromoacetamido analog, **I**, inactivated PGH_2 synthase, yielding K_i (inactivation) values of 2.7 μM and 0.93 mM, respectively. By contrast, mefenamic acid is a reversible inhibitor only while its corresponding bromoacetamido analog, **II**, inactivates PGH_2 synthase. Thus, incorporation of the bromoacetamido group into mefenamic acid converted a pure competitive inhibitor into an enzyme inactivator. Comparison of the half-lives for the enzyme at saturation indicated that while indomethacin and meclofenamic acid inactivated the holoenzyme within seconds, both bromoacetamido analogs, **I** and **II**, inactivated the enzyme over 3–5 h. This infers that the bromoacetamido analogs inactivate PGH_2 synthase by mechanisms that differ from those for indomethacin and meclofenamic acid.

Examination of the K_i(inhibition) and K_i(inactivation) values observed with the NSAIDs and **I** and **II** shows that two kinetically distinguishable complexes are formed with the enzyme. In the case of indomethacin and meclofenamic acid, high-affinity complexes are responsible for enzyme inactivation and low-affinity complexes are responsible for competitive inhibition. In the case of the bromoacetamido analogs, the situation is reversed, such that a high-affinity E·I complex is responsible for competitive inhibition while a low-affinity E·I complex is responsible for enzyme inactivation (Scheme 4).

An interesting feature of these experiments is that K_i(inhibition) and K_i(inactivation) can be distinguished in experiments in which O_2 uptake alone was measured, implying that the two complexes observed with each compound are related to the cyclooxygenase activity of the synthase. The existence of low- and high-affinity complexes for indomethacin and meclofenamic acid has been previously described (Rome & Lands, 1975; Lands & Hanel, 1983).

Parallel Inactivation of the Cyclooxygenase and Peroxidase Activities of PGH_2 Synthase. The ability of the affinity-labeling agents **I** and **II** to irreversibly inhibit the peroxidase activity of PGH_2 synthase was also investigated. Treatment of the holoenzyme with **I** and **II** caused a parallel inactivation of both the cyclooxygenase and peroxidase activities of PGH_2 synthase, whereas indomethacin and meclofenamic acid had no effect on the peroxidase activity (seen Figure 3A–C for representative data). The inability of these NSAIDs to block the peroxidase activity is in accord with previous studies

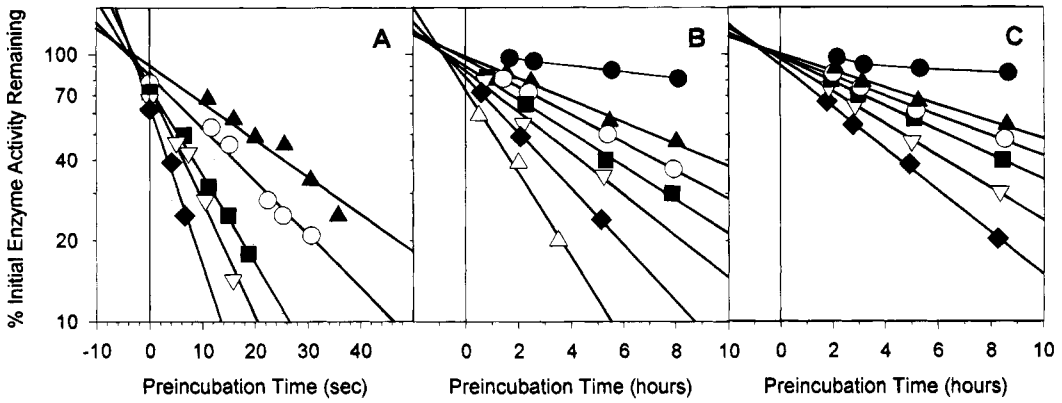


FIGURE 2: Time- and concentration-dependent inactivation of the cyclooxygenase activity of PGH_2 synthase by indomethacin (A), 4-BrAc-indomethacin (**I**) (B), and 4-BrAc-mefenamic acid (**II**) (C). Holo-PGH_2 synthase (40.0 μM) was incubated with various concentrations of (A) indomethacin [0.27 μM (▲), 0.54 μM (○), 1.08 μM (■), 2.7 μM (▽), and 5.4 μM (◆)], (B) 4-BrAc-indomethacin (**I**) [no drug (●), 0.27 mM (▲), 0.53 mM (○), 1.1 mM (■), 1.6 mM (▽), 2.13 mM (◆), and 2.67 mM (△)], and (C) 4-BrAc-mefenamic acid (**II**) [no drug (●), 1.28 mM (▲), 1.92 mM (○), 2.56 mM (■), 3.2 mM (▽), and 4.48 mM (◆)], in 100 μL of 100 mM Tris-HCl buffer (pH 8.0) at 25 °C. At the indicated times, 8 μL of the incubation mixture was withdrawn and diluted 75-fold into the cyclooxygenase assay system described in the text, and the amount of enzyme activity remaining was determined. Semilogarithmic plots of the percent initial enzyme activity remaining versus time are shown. By drawing tangents to the initial portion of each progress curve, the pseudo-first-order rate constants (k_{app}) were obtained. These data were then analyzed by the method of Kitz and Wilson (1962).

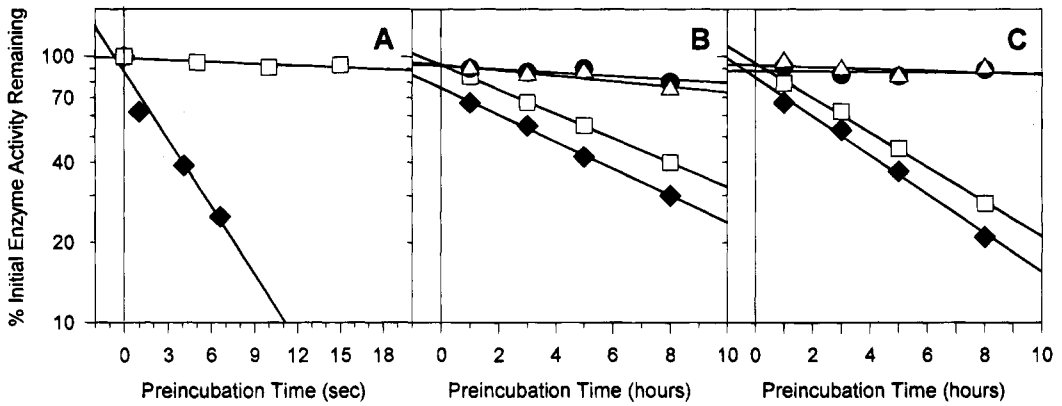
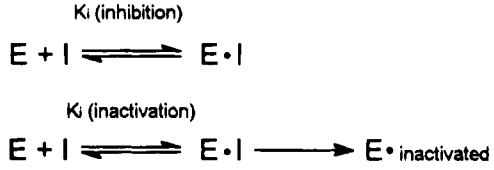


FIGURE 3: Parallel inactivation of the cyclooxygenase and peroxidase activities of PGH_2 synthase by 4-BrAc-indomethacin (**I**) and 4-BrAc-mefenamic acid (**II**). Holo-PGH_2 synthase (40.0 μM) was preincubated either with 10 μM indomethacin and the amount of cyclooxygenase activity (◆) was determined or with 1 mM indomethacin and the amount of peroxidase activity (□) was determined (A). Holo-PGH_2 synthase (40.0 μM) was preincubated with 4.0 mM **I**, and the cyclooxygenase activity remaining (◆) plus the peroxidase activity remaining (□) was determined. As a control, the cyclooxygenase activity (●) and peroxidase activity (△) were measured for holo-PGH_2 synthase in the absence of any drug (B). Holo-PGH_2 synthase (40.0 μM) was preincubated with 3.6 mM **II**, and the cyclooxygenase activity remaining (◆) plus the peroxidase activity remaining (□) was determined. As a control, the cyclooxygenase activity (●) and peroxidase activity (□) were measured for holo-PGH_2 synthase in the absence of any drug (C). In these experiments, the peroxidase activity was determined using method I. Semilogarithmic plots of the percent initial enzyme activity remaining versus time are shown.

Scheme 4: Interaction of NSAIDs and NSAID Analogs with PGH_2 Synthase[a]



[a] When I is indomethacin, K_i(inhibition) ≫ K_i(inactivation), and when I is a bromoacetamido analog, K_i(inactivation) ≫ K_i(inhibition).

(Vane, 1974; Rome & Lands, 1975). Thus, the ability of **I** and **II** to abolish both cyclooxygenase and peroxidase activities distinguishes these agents from classical NSAIDs, which can affect only the cyclooxygenase activity. Since **I** and **II** act as competitive inhibitors against arachidonic acid and, at the same time, abolish both cyclooxygenase and peroxidase activities, this would suggest that these agents may bind to more than one site in PGH_2 synthase.

Stoichiometry of Incorporation of **I** *and* **II** *into PGH_2 Synthase.* To determine the number of binding sites on PGH_2 synthase for the bromoacetamido analogs, **I** and **II**, the holoenzyme was incubated with either ^{14}C-radiolabeled **I** or **II** until both the cyclooxygenase and peroxidase activities were abolished. Excess label was removed, and radiolabeled PGH_2 synthase was isolated by SDS−PAGE. By determining the amount of protein applied to the gel along with the specific radioactivity of the ligand, a stoichiometry of either 2.1 mol of [^{14}C]BrAc-indomethacin (**I**) or 1.9 mol of [^{14}C]-BrAc-mefenamic Acid (**II**) per mole of inactivated PGH_2 synthase monomer was found in the recovered enzyme (see Figure 4). The ability to measure this stoichiometry after boiling the inactivated enzyme in SDS would indicate that the incorporation reflects true covalent bond formation. The stoichiometry of 2 would suggest that these analogs bind to at least two sites on PGH_2 synthase.

Our demonstration that **I** and **II** can act as competitive inhibitors against arachidonic acid implies that one site of

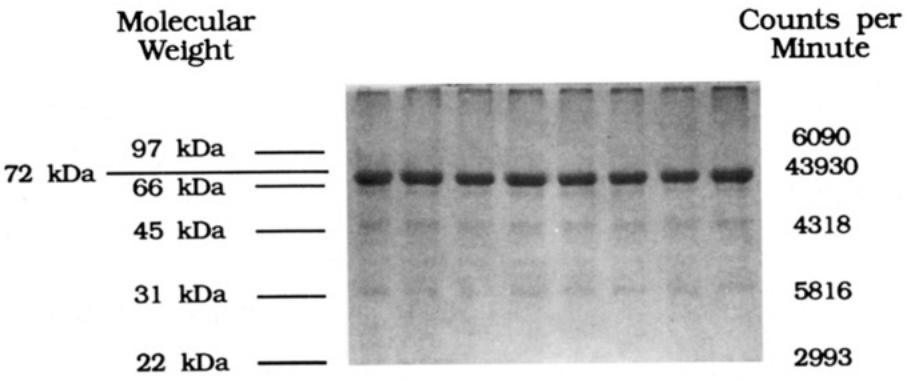


FIGURE 4: Stoichiometry of incorporation of 4-BrAc-indomethacin (**I**) and 4-BrAc-mefenamic acid (**II**) into PGH_2 synthase. Holo-PGH_2 synthase (40.0 μM) was incubated with either 3.0 mM [^{14}C]BrAc-indomethacin (**I**) or 2.0 mM [^{14}C]BrAc-mefenamic acid (**II**) for 48 h, and the samples were then boiled in SDS. The samples were then separated by SDS−PAGE, and the gels were stained with Coomassie Blue. Gel slices containing PGH_2 synthase were then incubated in NEN's Solvable and counted after the addition of Ecolite. By comparing the amount of protein applied to the gel with the specific activity of the ligand, a stoichiometry of either 2.1 mol of [^{14}C]BrAc-indomethacin (**I**) or 1.9 mol of [^{14}C]BrAc-mefenamic acid (**II**) per mole of inactivated PGH synthase monomer was obtained. Only the results obtained with **II** are shown.

covalent modification may correspond to the cyclooxygenase site. To ascertain this, holo-PGH_2 synthase (40.0 μM) was incubated with aspirin (100 μM) for 1 h, and the cyclooxygenase and peroxidase activities were assayed. This yielded PGH_2 synthase that was devoid of cyclooxygenase activity but retained peroxidase activity. This enzyme was then incubated with excess [^{14}C]BrAc-indomethacin (**I**) for 24 h. At the end of this time, the peroxidase activity was assayed and found to be absent. Excess label was removed, radiolabeled PGH_2 synthase was isolated by SDS−PAGE, and the stoichiometry of incorporation was determined. Inactivation of PGH_2 synthase containing only peroxidase activity gave a stoichiometry of 1.2 mol of [^{14}C]BrAc-indomethacin (**I**) per mole of inactivated enzyme. Since the stoichiometry of incorporation of **I** in the presence of aspirin was reduced to 1, this finding confirms that one of the two sites modified by the bromoacetamido analogs corresponds to the cyclooxygenase site.

Mefenamic Acid Protects Cyclooxygenase Activity but Not Peroxidase Activity against Inactivation by **I** *and* **II**. If one of the two sites labeled by **I** and **II** corresponds to the peroxidase site, then it should be possible to chemically prepare PGH_2 synthase with its cyclooxygenase activity intact while lacking peroxidase activity. In these experiments, holo-PGH_2 synthase (40.0 μM) was incubated with either **I** or **II** in the presence and absence of mefenamic acid for 24 h, and the remaining cyclooxygenase and peroxidase activities were determined after dialyzing out all drugs. Treatment of the holoenzyme with mefenamic acid protected cyclooxygenase activity but not peroxidase activity against inactivation by **I** and **II** (see Figure 5). Thus, PGH_2 synthase that had no peroxidase activity but retained cyclooxygenase activity was generated. Taken together, the ability of aspirin to reduce the stoichiometry of covalent modification observed with **I** from 2 to 1 and the ability of mefenamic acid to protect the cyclooxygenase site from inactivation by **I** and **II** provide evidence that PGH_2 synthase has two binding sites for these bromoacetamido analogs. These findings also suggest that of the two sites modified, one may correspond to the cyclooxygenase site and the other may correspond to the peroxidase site.

Since the ability to prepare PGH_2 synthase devoid of peroxidase activity has mechanistic implications (see Discussion), this finding was confirmed using a peroxidase assay

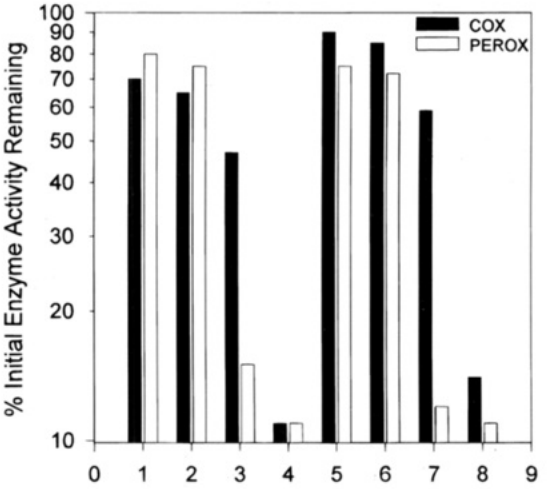


FIGURE 5: Mefenamic acid protects cyclooxygenase activity but not peroxidase activity against inactivation by 4-BrAc-indomethacin (**I**) and 4-BrAc-mefenamic acid (**II**). Holo-PGH_2 synthase (40.0 μM) was incubated with either 2.7 mM **I** or 4.5 mM **II** in the presence and absence of 100 μM mefenamic acid for 8 h [no drug, 1 and 5; 100 μM mefenamic acid, 2; 2.7 mM **I** and 100 μM mefenamic acid, 3; 2.7 mM **I**, 4; 120 μM mefenamic acid, 6; 4.5 mM **II** and 120 μM mefenamic acid, 7; 4.5 mM **II**, 8]. Aliquots were removed over time and diluted 75-fold into the cyclooxygenase (closed box) or peroxidase (open box) assay systems (method I), respectively, and the remaining enzyme activity was determined.

that measured peroxide bond cleavage (method II). PGH_2 synthase was incubated with saturating amounts of mefenamic acid and either **I** or **II**. The disappearance of peroxidase activity was monitored by measuring TMPD reduction (method I). All drugs were then removed by extensive dialysis. Enzyme treated in this manner had 70% of the cyclooxygenase activity of the control (untreated) enzyme, but it lacked the ability to cleave ethyl hydroperoxide to ethanol and water (see Table 2).

DISCUSSION

Bromoacetamido analogs of indomethacin and mefenamic acid, **I** and **II**, were originally synthesized as affinity-labeling agents to map the topography of NSAID binding sites (Askonas & Penning, 1991; Tang et al., 1994). In this study, the effects of **I** and **II** on homogeneous PGH_2 synthase from ram seminal vesicles were examined. Our findings have

Table 2: Generation of Peroxidase-Free PGH_2 Synthase

	cyclooxygenase activity			peroxidase activity: TMPD + H_2O_2			peroxidase activity: EtOOH			
time (h)	treated (nmol min^{-1})	control (nmol min^{-1})	%	treated (nmol min^{-1})	control (nmol min^{-1})	%	treated (nmol min^{-1})	control (nmol min^{-1})	%	% Cox/ % Pox
0	251 ± 6	249 ± 3	100	44 ± 3	44 ± 3	100	6.0 ± 0.3	6.1 ± 0.4	100	1
6	214 ± 9	231 ± 5	91	24 ± 3	40 ± 3	59	ND	ND	ND	1.54
12	179 ± 8	212 ± 4	86	8 ± 4	37 ± 4	22	ND	ND	ND	3.91
18	163 ± 4	201 ± 7	80	1 ± 1	34 ± 5	3	ND	ND	ND	26.6
24	138 ± 6	188 ± 9	73	0	32 ± 5	0	0	4.8 ± 0.5	0	∞

[a] ND, not determined. PGH_2 synthase (20.0 μM) was diluted in 0.5 mL of 100 mM Tris (pH 8.0) containing 1 mM phenol and 100 μM hematin. The cyclooxygenase activity was determined by measuring the oxygen consumption in the presence of arachidonic acid. The peroxidase activity was measured using either TMPD and H_2O_2 as cosubstrates (method I) or the peroxidative cleavage of EtOOH to ethanol and water (method II) (see Experimental Procedures). Mefenamic acid (150 μM) and 4-BrAc-indomethacin (**I**) (5.3 mM) were then added in DMSO (final concentration, 5%). Aliquots were removed over time and diluted 75-fold into the cyclooxygenase or peroxidase assay systems, respectively, and the enzyme activity remaining was determined. After the peroxidase assay (method I) had established that the peroxidase activity was abolished, the reaction mixture was dialyzed to remove all drugs. At the end of dialysis, the reaction mixture was reassayed for cyclooxygenase activity and peroxidase activity using methods I and II. The experiment was performed in triplicate and each assay was duplicated. Similar results were obtained for 4-BrAc-mefenamic acid (**II**) (data not shown).

implications concerning the number of E·I complexes that can form on cyclooxygenase, the number of NSAID binding sites present on the enzyme and their location, the isolation and sequencing of peptides that compose the drug binding site(s), and the catalytic mechanism.

Reversible versus Irreversible Inhibition of the Cyclooxygenase Activity of PGH_2 Synthase. Kinetic studies indicated that **I** and **II** act as competitive inhibitors of arachidonic acid, yielding high-affinity E·I complexes. Compounds **I** and **II** also act as time-dependent, irreversible inhibitors of the cyclooxygenase reaction of PGH_2 synthase via low-affinity E·I complexes. Since our kinetic experiments were conducted by measuring oxygen consumption, both E·I complexes are related to the cyclooxygenase reaction. Coincubation of PGH_2 synthase with mefenamic acid, a pure competitive inhibitor versus arachidonic acid, protected the cyclooxygenase activity against inactivation by **I** and **II**, implying that both the low- and high-affinity E·I complexes are mutually exclusive.

This is not the first example whereby NSAIDs can form two kinetically distinguishable E·I complexes that are mutually exclusive. NSAIDs containing an aryl halide and a carboxylic acid functionality (e.g., indomethacin and meclofenamic acid) inhibit PGH_2 synthase in this manner (Rome & Lands, 1975). They act as pure competitive inhibitors against arachidonic acid, yielding low-affinity E·I complexes, and as time-dependent inactivators, yielding high-affinity E·I complexes (Lands & Hanel, 1983; Kulmacz & Lands, 1985). These observations were confirmed in the present study. However, the complexes that form with **I** and **II** show some important differences that should be emphasized. Unlike indomethacin and meclofenamic acid, it is the low-affinity (E·I) complex that inactivates the enzyme. Second, this low-affinity complex results in covalent modification, yielding a half-life of 6 h for the enzyme at saturation. By contrast, indomethacin and meclofenamic acid cause rapid inactivation of the cyclooxygenase reaction, yielding half-lives in seconds, but do not cause covalent modification of the enzyme (Stanford et al., 1977). Clearly, the two mechanisms of inactivation are different. The long half-lives for the inactivation of synthase observed with **I** and **II** imply that covalent modification occurs via nucleophilic attack of the bromoacetamido group by an amino acid side chain of low reactivity rather than by a reactive functional group, e.g., the thiol group of a cysteine residue. What are the reasons for the formation of these kinetically distinct complexes? Kulmacz and Lands (1985) postulated that the time-dependent effect of indomethacin on PGH_2 synthase involved a drug-induced conformational change. Similarly, **I** and **II**, in the absence of arachidonic acid, may induce their own conformational change to yield an enzyme more prone to covalent modification.

Covalent Modification of the Cyclooxygenase and Peroxidase Sites. Compounds **I** and **II** form stable covalent bonds with PGH_2 synthase since the resultant bonds are resistant to boiling in SDS and permit the stoichiometry of incorporation to be determined by SDS−PAGE analysis. Our data show that **I** and **II** are the first NSAID analogs other than aspirin that can covalently modify PGH_2 synthase in a stable manner. These radiolabeled analogs could be used to isolate and sequence peptides that compose the NSAID binding site(s). Unlike classical NSAIDs, which affect only the cyclooxygenase activity of PGH_2 synthase, **I** and **II** caused slow, time-dependent inactivation of both the cyclooxygenase and peroxidase activities of the synthase in a parallel manner. These events are accompanied by the incorporation of 2 mol of inactivator bound per mole of synthase monomer. This stoichiometry suggests the modification of two distinct sites. Aspirin protection experiments that reduced the stoichiometry to 1 imply that one site corresponds to the cyclooxygenase site. Since mefenamic acid can protect the cyclooxygenase site while **I** and **II** can still abolish the peroxidase activity, the second site may correspond to the peroxidase site. The ability to selectively modify either the cyclooxygenase or peroxidase site implies that these sites are separate, a finding that is supported by the X-ray structure of PGH_2 synthase (Picot et al., 1994).

Mechanistic Considerations. When PGH_2 synthase was preincubated with mefenamic acid, and **I** or **II** was used to abolish the peroxidase activity, the removal of all drugs by dialysis gave a preparation of PGH_2 synthase that could perform the cyclooxygenase reaction, but lacked the ability to cleave ethyl hydroperoxide to ethanol and water. The ability to chemically prepare PGH_2 synthase devoid of peroxidase activity has mechanistic implications. The mechanism proposed by Ruf and co-workers (Dietz et al., 1988; Ruf et al., 1993) requires a peroxidase step in which there is heterolytic cleavage of a hydroperoxide with

concomitant change in the oxidation state of the iron in heme from Fe^{3+} to Fe^{5+}. This in turn generates the TyrO•, which initiates the cyclooxygenase reaction by the stereospecific abstraction of the *pro-S* hydrogen from the allylic C-13 position of arachidonic acid (Hamburg & Samuelsson, 1967). The preparation of PGH_2 synthase that maintains cyclooxygenase activity in the absence of peroxidase activity does not support this mechanism. Previous studies have indicated that such preparations of the enzyme may exist. Thus, Shimokawa and Smith (1991) were able to generate PGH_2 synthase mutants (His386Gln and His386Ala) devoid of peroxidase activity, while manganese and cobalt protoporphyrin IX–PGH_2 synthases (Lassmann et al., 1991; Smith & Marnett, 1991) retained cyclooxygenase activity with only residual peroxidase activity. These enzyme preparations question mechanisms of PGH_2 synthase catalysis in which a peroxidase step must proceed the cyclooxygenase step.

Previously, Hemler and Lands (1980) had proposed a mechanism for PGH_2 synthase catalysis in which the reaction was driven by the formation of a hydroperoxy radical ROO•, which was produced at the expense of reducing Fe^{3+} to Fe^{2+}. Since the bromoacetamido analogs **I** and **II** used in this study block the peroxidase site, they would presumably block the interaction of a hydroperoxide with the iron and prevent the formation of Fe^{2+} and the peroxy radical. The ability to generate cyclooxygenase activity in the absence of measurable peroxidase activity could be explained if the peroxy radical is formed independently of the peroxidase site. Peroxy radicals are produced during the bis-dioxygenation of arachidonic acid that occurs at the cyclooxygenase site, and these could act as oxidants to generate either a tyrosyl or an alternate radical to propagate the reaction. Marnett and co-workers have shown that when Mn^{3+}-protoporphyrin IX synthase is incubated with arachidonic acid, it catalyzes radical formation and yet has less than 0.8% peroxidase activity (Odenwaller et al., 1992). These observations create the precedent for a peroxidase-independent pathway for radical formation that occurs at the cyclooxygenase site. Our data would be consistent with the formation of a peroxy radical at the cyclooxygenase site. Since the recently solved X-ray structure of PGH_2 synthase shows physically distinct peroxidase and cyclooxygenase sites (Picot et al., 1994), the generation of a peroxy radical in the cyclooxygenase site could occur uninfluenced by events at the peroxidase site.

ACKNOWLEDGMENT

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