Synthesis and Use of Iodinated Nonsteroidal Antiinflammatory Drug Analogs as Crystallographic Probes of the Prostaglandin H_2 Synthase Cyclooxygenase Active Site[†,‡]

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ABSTRACT: The cyclooxygenase activity of the membrane protein prostaglandin H_2 synthase isoform 1 (PGHS-1) is the target of the nonsteroidal antiinflammatory drugs (NSAIDs). The X-ray crystal structures of PGHS-1 in complex with the NSAIDs flurbiprofen and bromoaspirin have been determined previously [Picot, D., et al. (1994) *Nature 367*, 243−249; Loll, P. J., et al. (1995) *Nat. Struct. Biol. 2*, 637−643]. We report here the preparation and characterization of novel potent iodinated analogs of the NSAIDs indomethacin and suprofen, as well as the refined X-ray crystal structures of their complexes with PGHS-1. The PGHS−iodosuprofen complex structure has been refined at 3.5 Å to an *R*-value of 0.189 and shows the suprofen analog to share a common mode of binding with flurbiprofen. The PGHS−iodoindomethacin complex structure has been refined at 4.5 Å to an *R*-value of 0.254. The low resolution of the iodoindomethacin complex structure precludes detailed modeling of drug−enzyme interactions, but the electron-dense iodine atom of the inhibitor has been unambiguously located, allowing for the placement and approximate orientation of the inhibitor in the enzyme's active site. We have modeled two equally likely binding modes for iodoindomethacin, corresponding to the two principal conformers of the inhibitor. Like flurbiprofen, iodosuprofen and iodoindomethacin bind at the end of the long channel which leads into the enzyme active site. Binding at this site presumably blocks access of substrate to Tyr-385, a residue essential for catalysis. No evidence is seen for significant protein conformational differences between the iodoindomethacin and iodosuprofen of flurbiprofen complex structures.

Prostaglandin H_2 synthase (PGHS,[1] also known as cyclooxygenase; EC 1.14.99.1) catalyzes the two-step conversion of arachidonic acid to prostaglandin G_2 and thence to prostaglandin H_2, the first committed steps in the biosynthesis of a class of potent hormones which includes various prostaglandins, prostacyclin, and thromboxanes [for recent reviews, see Garavito et al. (1994) and Smith & Marnett (1994)]. While PGHS catalyzes both steps of this catalytic process (known as the cyclooxygenase and peroxidase steps, respectively), the reactions are carried out in two spatially distinct active sites. The cyclooxygenase activity is inhibited by the class of compounds known as nonsteroidal antiinflammatory drugs (NSAIDs), which includes the commercially and pharmacologically important agents aspirin, ibuprofen, naproxen, and indomethacin (Loll & Garavito, 1994). Arachidonic acid metabolites derived from prostaglandin G_2 are known to be powerful mediators of the inflammatory response, and the NSAIDs achieve their antiinflammatory and antipyretic effects chiefly through blocking prostaglandin G_2 formation via inhibition of PGHS.

At least two isoforms of PGHS are known to be expressed in mammalian tissues. The first, PGHS-1, is expressed constitutively in most tissue types and appears to be the product of a "housekeeping" gene, supporting the low levels of prostanoid biosynthesis required for maintaining homeostasis. In contrast, the second isoform, PGHS-2, displays a radically different tissue distribution and pattern of expression. While constitutively expressed in brain cells, PGHS-2 can be rapidly induced, by cytokines and other mitogens, to high levels of expression in a limited number of cell types, including synovial cells, fibroblasts, macrophages, and monocytes. This transient PGHS-2 expression is elicited by specific inflammatory stimuli and is responsible for the synthesis of large quantities of prostanoids seen to occur during inflammation (Hla et al., 1993; Herschman, 1994). Hence, drug therapy aimed at reducing inflammation should

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[‡] The final refined coordinates of all structures presented herein have been deposited in the Protein Data Bank (entry names: 1PGE, 1PGF, 1PGG).

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[1] Abbreviations: PGHS, prostaglandin H2 synthase; β-OG, octyl β-D-glucopyranoside; NSAID, nonsteroidal antiinflammatory drug; iodosuprofen, *p*-(2′-iodo-5′-thenoyl)hydrotropic acid (**1**); iodoindomethacin, 1-(4-iodobenzoyl)-5-methoxy-2-methylindole-3-acetic acid (**2**); bromoaspirin, 2-(bromoacetoxy)benzoic acid; Et_2O, diethyl ether; HOAc, acetic acid; F_o, observed structure factor amplitude; F_c, calculated structure factor amplitude; COX, cyclooxygenase; MIR, multiple isomorphous replacement; NCS, noncrystallographic symmetry.

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be targeted toward isoform 2. Most NSAIDs currently in clinical use, however, are known to inhibit both isoforms of PGHS with little selectivity, and during extended therapy many NSAIDs cause ulcerogenic side effects most likely due to PGHS-1 inhibition in the stomach. The two isoforms have been shown to differ pharmacologically, and it should prove possible to exploit these differences to target PGHS-2, thereby decoupling the inhibition of inflammatory prostanoid biosynthesis from the inhibition of background levels of prostanoid biosynthesis required for normal physiological functioning (Isakson et al., 1995; Smith et al., 1994; Vane & Botting, 1995). All the NSAIDs are generally thought to target the cyclooxygenase activity of PGHS; however, the mechanisms by which they act vary widely. Some inhibit by covalently modifying the enzyme, some are competitive with respect to arachidonic acid, and some are slow-binding inhibitors. Information about the structural basis of NSAID binding by PGHS-1 and PGHS-2 should help to clarify why different compounds act by different mechanisms and may prove valuable in this process of designing isoform-specific inhibitors.

PGHS is an integral membrane protein that requires solubilization with detergent in order to remove it from the lipid bilayer and to purify it. The 3.5 Å structure of the complex of ovine PGHS-1 with flurbiprofen has been determined from crystals prepared in the presence of the nonionic detergent octyl β-D-glucopyranoside (β-OG) (Picot et al., 1994), and the resolution of this structure has recently been extended to 3.1 Å (Picot, Loll, and Garavito, unpublished). This structure opens the door for rigorous structural analysis of different PGHS−NSAID complexes. PGHS crystals, however, are very fragile, difficult to reproduce, exquisitely sensitive to changes in temperature and mother liquor composition, and consist of roughly 72% solvent by volume. In many cases, these factors conspire to limit the available resolution. In addition, the diffraction quality of the crystals obtained varies significantly as one changes the inhibitor bound to the enzyme.

Because of these experimental difficulties, we have probed the cyclooxygenase active site with NSAID analogs labeled with heavy atoms such as iodine or bromine. We reason that even in cases where crystals of PGHS−NSAID complexes do not diffract to near 3 Å, the electron-dense halogen will be readily located in difference Fourier maps, enabling us to position and orient the NSAID analog in the active site. Such a strategy for crystallographic analysis of enzyme-ligand interactions is not new (Olsen et al., 1975; Abdallah et al., 1975, 1979) and allowed us to unambiguously assign density observed in the initial 3.5 Å MIR maps to flurbiprofen and dock that compound into the active site (Picot et al., 1994) and to analyze the structural basis of aspirin activity by examining the structure of PGHS inactivated by a brominated aspirin analog (Loll et al., 1995a). Here, we report the continued application of this strategy to analyze the structures of two complexes of PGHS with iodinated NSAID analogs: A complex of PGHS and a suprofen analog refined at 3.5 Å resolution and a complex of PGHS and an indomethacin analog determined and refined at 4.5 Å resolution.

EXPERIMENTAL PROCEDURES

Materials. Arachidonic acid was obtained from Cayman Chemicals (Ann Arbor, MI). Detergents were obtained from Calbiochem (β-OG and decyl β-D-maltoside; La Jolla, CA) and Pierce (Tween-20; Rockford, IL). Suprofen and indomethacin were obtained from Sigma (St. Louis, MO). Other reagents and PEG-4000 were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Fluka (Ronkonkoma, NY) and were of the highest quality commercially available. NMR spectra were acquired at room temperature with Varian XL-300 and General Electric GN Omega 300 spectrometers. DEPT and COSY experiments were performed using standard pulse sequences. Electron impact mass spectra (EIMS) were recorded at 70 eV with a double focusing VG analytical mass spectrometer. Samples for EIMS were introduced by direct insertion with solid probes. Mass measurements for the high-resolution mass spectra (HRMS) were done by software peak matching using perfluorokerosene (PFK) as the reference standard.

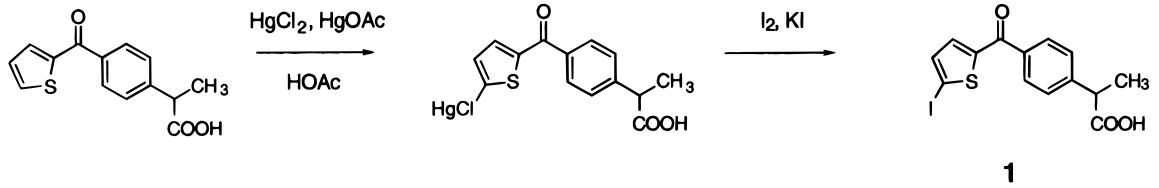
Enzyme Purification and Crystallization. PGHS used for kinetic analyses was purified by the method of Mevkh et al. (1985) and stored frozen at −70 °C in 100 μL aliquots. Enzyme used for crystallization experiments was purified as described (Garavito et al., 1995). All crystals were grown by the hanging drop method. Crystals of the iodosuprofen complex were prepared by dialyzing a 12 mg/mL solution of PGHS versus a buffer containing 20 mM sodium phosphate, pH 6.7, 80 mM NaCl, 0.6% (w/w) β-OG, and 100 μM iodosuprofen overnight at 4 °C. Sufficient quantities of a 40% (w/w) stock solution of PEG-4000 in water were added to the protein to bring it to 2% PEG (w/w); 5 μL drops of the protein solution were placed over wells containing 1 mL of 6.5% PEG-4000, 65 mM sodium phosphate, and 260 mM NaCl and incubated at 18 °C. Large single crystals appeared within 3 weeks.

Crystals prepared by cocrystallization of PGHS and iodoindomethacin invariably diffracted very poorly, if at all, and attempts to soak iodoindomethacin into crystals grown in the absence of inhibitor were also not successful. The formation of the flurbiprofen−PGHS complex is reversible, albeit slowly, with a $t_{1/2}$ of several hours. Hence, crystals of the iodoindomethacin complex were prepared by ligand exchange as follows: Crystals of the flurbiprofen complex, grown as described (Garavito et al., 1995), were transferred to a soak buffer containing 10% PEG-4000, 150 mM NaCl, 0.5% β-OG, 1 mM iodoindomethacin, and 20 mM sodium phosphate at pH 6.7; after several washes, the crystals were incubated overnight at 18 °C prior to data collection.

Enzyme Assays. PGHS cyclooxygenase activity was measured by monitoring oxygen consumption at 25 °C essentially as described (Kulmacz & Lands, 1987). Briefly, enzyme was incubated for 90 s at a concentration of 50 nM in a buffer containing 0.1 M Tris, pH 8.0, 1 mM phenol, 500 nM hemin, and the desired concentration of inhibitor, after which the reaction was initiated by addition of arachidonic acid (18 nmol per 600 μL, 30 μM). Measurements were carried out with an Instech Clark electrode (Plymouth Meeting, PA) and a Yellow Springs Instrument Co. biological oxygen monitor.

*Preparation of p-(2′-Iodo-5′-thenoyl)hydrotropic Acid (**1**).* The synthesis of **1** was adapted from the synthesis of 2-iodo-5-benzothienone (Weitkamp & Hamilton, 1937); see Scheme 1. Racemic suprofen (3.40 g, 13.1 mmol), $HgCl_2$ (1.79 g, 6.6 mmol), $Hg(CH_3CO_2)_2$ (2.10 g, 6.6 mmol), and acetic acid (26.5 mL) were refluxed for 75 min. The precipitate which formed upon cooling was washed with brine, water, and

Scheme 1: Preparation of *p*-(2′-Iodo-5′-thenoyl)hydrotropic Acid (Iodosuprofen, **1**)



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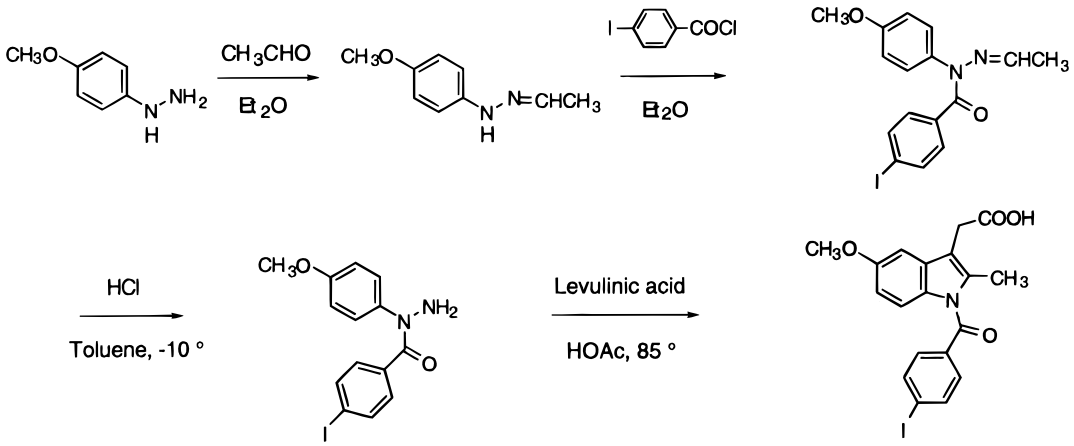
Scheme 2: Preparation of 1-(4-Iodobenzoyl)-5-methoxy-2-methylindole-3-acetic Acid (Iodoindomethacin, **2**)



2

ethanol and then lyophilized, yielding 1.48 g of creamy white powder. The organomercurial (1.37 g, 2.8 mmol) was suspended in 5 mL of water. Iodine (5.6 mL of 0.5 M I_2 in aqueous 3 M KI, 2.8 mmol) was added, and the mixture was swirled in a boiling water bath for 10 min, resulting in significant loss of the yellow color, after which it was cooled in an ice bath. The resulting gummy precipitate was washed with 3 M KI and water and then dissolved in ethanol. Evaporation of the ethanol yielded a brown viscous oil, which set to a glass overnight at 4 °C. Lyophilization of this material yielded 0.46 g of **1** (1.2 mmol, 9.8% yield). Recrystallization from pentane/ethyl acetate gave off-white needles, mp 132 °C. ^{1}H-NMR (DMSO-d_6, 300 MHz): ∂ 12.64 (1 H, br s, H-8), 7.94 (2 H, d, J = 7.9, H-3, H-5), 7.67 (1 H, d, J = 3.8, H-4′), 7.62 (2 H, d, J = 8.1, H-2, H-6), 7.55 (1 H, d, J = 3.8, H-3′), 3.96 (1 H, q, J = 7.2, H-7), 1.55 (3 H, d, J = 7.2, H-9). EI-HRMS (70 eV): *m/z*, $[M]^+$ 385.9473682 (calcd 385.9473681 for $C_{14}H_{11}IO_3S$). Anal. Calcd: C, 43.54; H, 2.87; I, 32.86; O, 12.43. Found: C, 43.18; H, 2.69; I, 34.21; O, 12.51.

*Preparation of 1-(4-Iodobenzoyl)-5-methoxy-2-methylindole-3-acetic Acid (**2**).* The synthesis of **2** was modeled after a reported adaptation of the Fischer indole synthesis (Yamamoto, 1967, 1968); see Scheme 2. *p*-Methoxyphenylhydrazine (12.3 g, 89 mmol; freshly prepared from the hydrochloride) was suspended in 250 mL of Et_2O on ice under N_2. To this was slowly added dropwise acetaldehyde (5.5 mL, 97 mmol) in Et_2O (5 mL). After 1 h the mixture was allowed to warm to room temperature, the Et_2O was evaporated, and the resulting dark orange oil was distilled under vacuum. The distillate (10.3 g, 63 mmol) was dissolved in 45 mL of Et_2O and cooled on ice. To this was added *p*-iodobenzoyl chloride (16.8 g, 63 mmol) in 50 mL of Et_2O with vigorous stirring. The resulting precipitate was washed with water and lyophilized (17.4 g, 44 mmol). The powder was dissolved in a mixture of methanol and toluene (1:9 v/v, 400 mL) and cooled in an ethylene glycol/dry ice bath; HCl was then bubbled through the solution for 2 h. The crystalline precipitate (13.2 g, 33 mmol) was washed, dried, and suspended in 200 mL of acetic acid at 80 °C; levulinic acid (4 mL, 39 mmol) was added, and the temperature was maintained between 80 and 90 °C for 3 h. The mixture was then cooled and poured into water. The resulting yellow/brown powder was washed exhaustively and lyophilized, yielding crude **2** (11.6 g, 26 mmol), mp 200 °C (29% yield). Recrystallization from 2-ethoxyethanol/water gave yellow needles, mp 204.5 °C. ^{1}H-NMR (DMSO-d_6, 300 MHz): ∂ 12.39 (1 H, s, H-12), 7.97 (2 H, d, J = 8.4, H-3′, H-5′), 7.43 (2 H, d, J = 8.1, H-2′, H-6′), 7.04 (1 H, d, J = 2.5, H-4), 6.93 (1 H, d, J = 8.9, H-7), 6.72 (1 H, dd, J = 9.0, 2.5, H-6), 3.76 (3 H, s, 5-OCH_3), 3.67 (2 H, s, H-11), 2.22 (3 H, s, H-10). EI-HRMS (70 eV): *m/z* $[M]^+$ 449.0124102 (calculated 449.0124101 for $C_{19}H_{16}NO_4I$). Anal. Cald: C, 50.80; H, 3.59; N, 3.12; O, 14.24; I, 28.25. Found: C, 50.70; H, 3.53; N, 3.10; O, 14.27; I, 29.06. The structure of **2** was verified by single crystal X-ray diffraction analysis (Loll et al., 1995b).

Diffraction Data Collection. Diffraction data sets were collected with the Enraf-Nonius FAST (Fast scanning Area Sensitive TV detector) area detector mounted on an Elliott GX-21 rotating anode X-ray generator producing Cu Kα radiation. The iodosuprofen complex data set was assembled from two crystals, while the iodoindomethacin complex data set was taken from a single crystal. Crystals were maintained at 4 °C throughout data collection. Individual data scans were integrated and profile-fit using the MADNES and PROCOR software packages (Messerschmidt & Pflugrath, 1987; Kabsch, 1988) and were combined and scaled by the ROTOVATA and AGROVATA programs from the CCP4 program suite (Collaborative Computational Project, No. 4, 1994). Data collection statistics are summarized in Table 1.

Refinement of the Iodosuprofen−PGHS Complex Structure. The starting protein model was derived by removing

Table 1: Data Collection and Refinement Statistics

	iodosuprofen	iodoindomethacin
space group	*I*222	*I*222
unit cell dimensions (Å)		
a	99.7	99.2
b	209.9	209.0
c	234.3	232.4
data collection statistics		
resolution (Å)	15.0−3.5	15.0−4.5
no. of observations	69965	15439
no. of unique reflections	28057	11169
R_{merge}	0.104	0.102
completeness (%)	90.9	79.2
final refinement parameters		
no. of non-hydrogen atoms[a]	2 × 4616	2 × 4602
resolution (Å)	8.0−3.50	8.0−4.5
R-value	0.189	0.254 (*cis*); 0.254 (*trans*)
no. of reflections	23300 ($F > 1\sigma$)	8235 ($F > 1\sigma$)
free *R*-value	0.231	0.267 (*cis*); 0.264 (*trans*)
no. of reflections	2574 ($F > 1\sigma$)	945 ($F > 1\sigma$)
completeness in range (%)	90.5	76.7
rms deviation from ideal geometry of final models[b]		
bond lengths (Å)	0.011	0.015
bond angles (deg)	1.77	1.99
dihedral angles (deg)	23.7	24.1
improper angles (deg)	1.53	1.73

[a] Strict 2-fold noncrystallographic symmetry imposed. [b] Values identical for both iodoindomethacin complex models.

all inhibitor, carbohydrate, and detergent molecules from the 3.1 Å refined PGHS−flurbiprofen structure (Picot et al., unpublished); individual refined *B*-values for each atom were retained without further refinement. The asymmetric unit of the crystal contains two molecules of PGHS which are related by a 2-fold noncrystallographic symmetry (NCS) axis, and the model was constrained to obey strict NCS throughout the course of the refinement. The starting model yielded an *R*-value of 0.50 for all data between 8 and 3.5 Å. It was subjected to rigid body refinement against the diffraction data using X-PLOR (Brunger, 1992), lowering the *R*-value to 0.242 (free $R = 0.247$). At this point, $2F_o - F_c$ and $F_o - F_c$ maps were calculated and revealed clear density for four *N*-acetylglucosamine residues and one β-OG molecule bound to each PGHS monomer. These features were observed in the 3.1 Å flurbiprofen complex structure but were omitted from the rigid body refinement and the subsequent map calculation; their appearance in the maps provides a good check on the quality of the phases. These maps also show clear density for the iodosuprofen inhibitors within each monomer, including extremely strong density for the iodine atoms (see Figure 2). All of the strong features observed in the $F_o - F_c$ map are accounted for by the carbohydrate, detergent, and inhibitor. These molecules were incorporated into the model in an iterative manner, interspersed with cycles of conjugate gradient positional refinement, refinement of overall anisotropic *B*-values, and model inspection. The refinement was judged to have converged after the *R*-value for reflections between 8 and 3.5 Å stabilized at 0.189 (free $R = 0.231$). An $F_o - F_c$ map calculated at this stage shows no features greater than 4 standard deviations; the density which is present at lower contour levels is randomly distributed and suggests no alterations to the model. This model has been deposited in the Protein Data Bank as entry 1PGE.

Refinement of the Iodoindomethacin−PGHS Complex Structure. The refinement of this structure began with the identical starting model as did the iodosuprofen structure, the 3.1 Å PGHS−flurbiprofen structure from which the flurbiprofen molecule, carbohydrate, and bound detergent had been removed. Before any refinement, this model yielded an *R*-value of 0.37 for all data between 8 and 4.5 Å. As with iodosuprofen, the two molecules of PGHS in the asymmetric unit were constrained to obey strict NCS during the refinement. Rigid body refinement and refinement of an overall anisotropic ΔB reduced the *R*-value to 0.282 (free $R = 0.285$). An $F_o - F_c$ map calculated at this stage revealed good electron density for the same four *N*-acetylglucosamine molecules per PGHS monomer observed in the other PGHS complex structures, providing an independent check on the quality of the calculated phases. The asymmetric unit of this $F_o - F_c$ map contains two strong peaks corresponding to the two iodine atoms, as well as weak and discontinuous density in other parts of the two active sites. These weaker features are presumably derived from the light atoms of the inhibitor. No other significant density was observed above the 3σ level. After the carbohydrate was added to the model, the iodoindomethacin molecule was placed by fixing the iodine at the site of high electron density and building the remainder of the molecule by hand. Two possible orientations of the inhibitor were identified, one of which requires the *cis* conformer of the inhibitor and the other the *trans* (see Figure 4). Each of these models was subjected to rigid body refinement, allowing the protein and inhibitor to move independently, which reduced the *R*-value to 0.276 (*trans*) and 0.277 (*cis*); free $R = 0.277$ and 0.274, respectively. While some bad contacts between the protein and the inhibitor persisted after these refinements, it appeared that these could be alleviated by small side chain motions in the active site. Accordingly, the following refinement protocol was devised. A core set of atoms (set 1; 570 atoms) comprising the inhibitor and all protein atoms within 8 Å of the inhibitor were subjected to positional refinement by conjugate gradient minimization. In addition, a 3 Å thick shell of atoms was defined around the atoms in set 1 and denoted set 2 (715 atoms); atoms in this shell were allowed to move but were made subject to harmonic restraints tethering them to their original positions. The remaining 4332 atoms were fixed. Coordinates for atoms in sets 1 and 2 represent 3855 degrees of freedom, which may be compared with the 8235 reflections in the working set and 945 reflections in the test set. After 300 cycles of this conjugate gradient refinement, the *R*-value dropped to 0.254 for the *trans* model (free $R = 0.264$) and to 0.254 for the *cis* model (free $R = 0.267$). These models have been deposited in the Protein Data Bank as entries 1PGF (*cis*) and 1PGG (*trans*).

RESULTS

PGHS Inhibition by Iodinated NSAID Analogs. Suprofen and indomethacin both show time-dependent inhibition of the cyclooxygenase activity of PGHS (Kulmacz & Lands, 1985; Loll, unpublished observations). We have verified that iodosuprofen and iodoindomethacin also display similar time-dependent inhibition (Loll, unpublished observations) but have not determined the kinetic constants. Figure 1 shows a comparison of cyclooxygenase inhibition mediated by iodosuprofen and iodoindomethacin with the inhibition

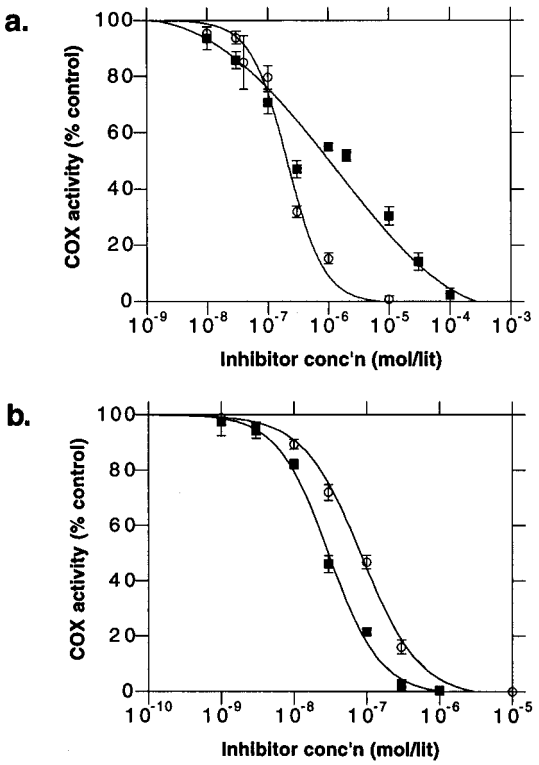


FIGURE 1: Comparison of cyclooxygenase inhibition by the parent compounds and by the iodinated analogs. PGHS was preincubated with inhibitor and oxygen consumption was measured as described in the Experimental Procedures. Enzyme activity is expressed as a percentage of the activity of control enzyme not incubated with inhibitor. Standard deviations were calculated from three to six independent measurements for each data point. (a) Suprofen vs iodosuprofen. Open circles refer to suprofen and filled squares to iodosuprofen. IC_{50} values: suprofen, 0.20 μM; iodosuprofen, 1.0 μM. (b) Indomethacin vs iodoindomethacin. Open circles refer to indomethacin and filled squares to iodoindomethacin. IC_{50} values: indomethacin, 83 nM; iodoindomethacin, 29 nM.

observed with the parent compounds. While it is evident from the figure that the introduction of the iodine atom onto the thiophene ring of suprofen has a slight deleterious effect on binding, increasing the IC_{50} value from 0.20 to 1.0 μM, the iodinated analog is still a highly effective inhibitor of the cyclooxygenase activity. In contrast, replacement of the *p*-chloro substituent of the phenyl ring of indomethacin with an iodine actually increases the potency of the compound, reducing the IC_{50} from 83 to 29 nM.

Refined Structures. Refinement statistics for the two PGHS complex structures are given in Table 1. Both show reasonable agreement statistics and good geometry, consistent with the resolution range of the available data. Luzzati analysis gives an estimate of the rms coordinate error for the PGHS−iodosuprofen complex structure of 0.3 Å. The lower resolution available for the PGHS−iodoindomethacin structure precludes use of the Luzzati method for estimating coordinate error. Both structures benefited from the use of the protein coordinates from the PGHS−flurbiprofen complex refined at 3.1 Å (Picot et al., unpublished). The higher resolution of the PGHS−flurbiprofen complex structure yielded a higher observable to parameter ratio, allowing for the refinement of restrained individual atomic *B*-values. These refined *B*-values were retained for the protein atoms in the PGHS complex structures with iodosuprofen and iodoindomethacin without further refinement. In both cases, this approach yielded better agreement with the data than the use of a single overall *B*-value, as evidenced by *R*- and free *R*-values after the initial cycles of rigid body refinement using the protein alone. The rms difference in C_α positions between the flurbiprofen and iodosuprofen structures is 0.19 Å. This small value and the low *R*-values obtained throughout the iodosuprofen refinement (particularly in the early stages) suggest that the protein structures in the flurbiprofen and iodosuprofen complexes do not differ significantly at this resolution.

Since the low resolution of the data obtained for the PGHS−iodoindomethacin complex does not allow for unrestricted refinement of the atomic positions, the protein structure in the model of this complex is essentially identical to the starting model save for the immediate vicinity of the drug. The *R*-values indicate that the protein models derived from the PGHS-flurbiprofen complex fit the iodoindomethacin data reasonably well. These *R*-values are somewhat higher than that obtained with iodosuprofen; this may reflect the inability of the refinement algorithm to find low minima when working with-low resolution data, or it may reflect genuine differences between the protein structure in the presence of flurbiprofen and in the presence of iodoindomethacin. It is also likely that the iodoindomethacin did not effect complete displacement of the flurbiprofen and that the structure is heterogeneous in the cyclooxygenase active site region, with some molecules containing iodoindomethacin and others containing flurbiprofen and/or no bound inhibitor. Finally, since we can fit the light atoms of the inhibitor into the active site in two orientations, we cannot rule out the possibility of conformational heterogeneity within the inhibitor.

Iodosuprofen−PGHS Complex Structure. Iodosuprofen binds to PGHS at the top of the long cyclooxygenase channel leading from the membrane binding surface to the cyclooxygenase active center (Figure 5). Flurbiprofen has already been found to bind in this site (Picot et al., 1994), and no significant differences are observed between the positions and orientations of active site side chains in the flurbiprofen and iodosuprofen complex structures. This comes as no surprise, given the strong similarity between the two inhibitors.

The iodosuprofen is oriented with its carboxylate group outermost; that is, it appears to have entered the narrow channel thiophene ring first. Side chains lining the channel below the inhibitor, including Tyr-355 and Arg-120, close around the inhibitor and render it inaccessible to solvent. We calculate that greater than 98% of the surface area of the inhibitor is buried in the complex. As is the case with flurbiprofen, the carboxylic acid moiety of iodosuprofen forms a salt bridge with Arg-120, the two carboxylate oxygen atoms of the inhibitor lying 2.8 and 3.0 Å, respectively, from one of the guanidino nitrogens of the arginine (Figure 2). In addition, one carboxylic acid oxygen of iodosuprofen lies 2.9 Å from the phenolic hydroxyl of Tyr-355, evidently forming a hydrogen bond. A hydrogen bond also appears to be formed between the carbonyl oxygen of the inhibitor's ketone moiety and the side chain hydroxyl of Ser-530: the two atoms are separated by 3.0 Å and display a favorable geometry for hydrogen bonding. These are the only polar interactions the iodosuprofen molecule is observed to make with PGHS.

The basis of the enzyme's stereoselectivity for the *S*-stereoisomer of iodosuprofen is clear from the structure and

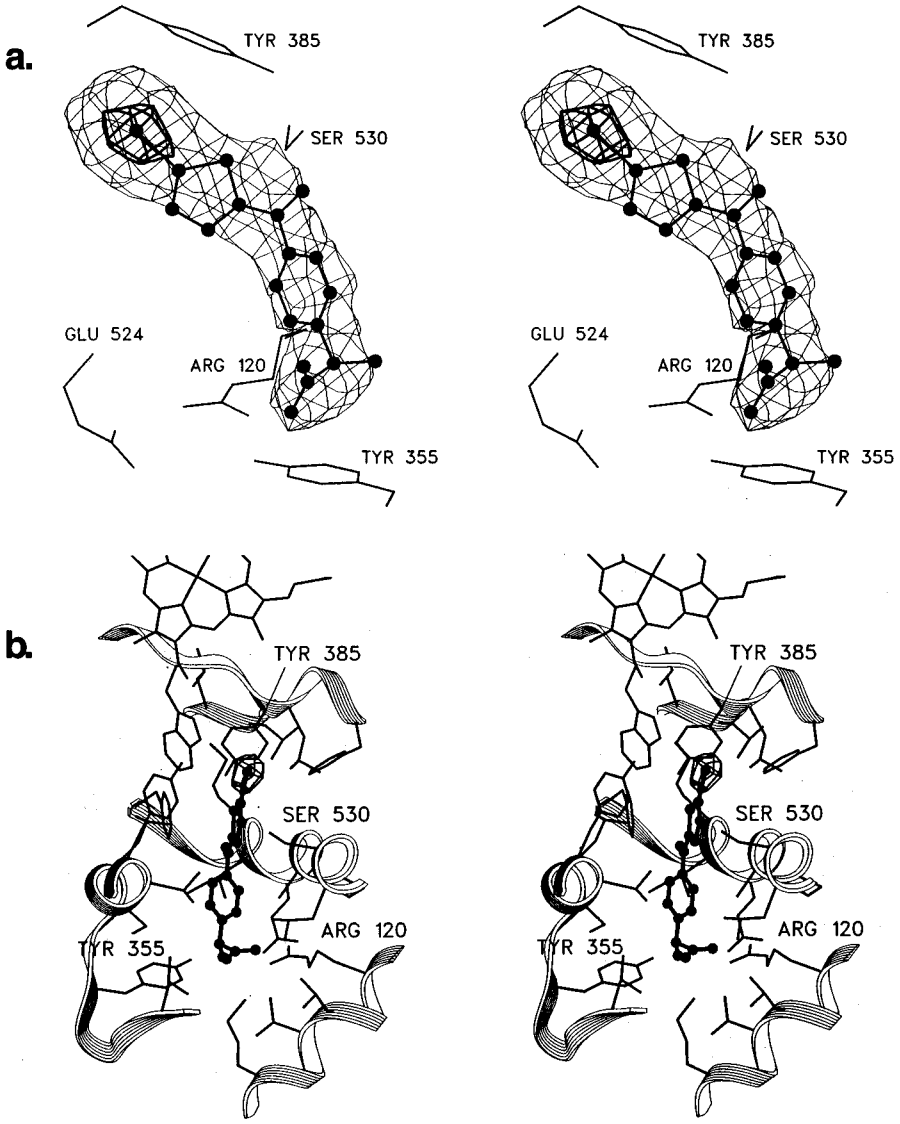


FIGURE 2: Stereoviews of the difference electron density corresponding to the iodosuprofen molecule bound to PGHS. The map shown is an $F_o - F_c$ difference map calculated after rigid body refinement of the starting model (see Experimental Procedures) against the iodosuprofen data. The final refined model is shown superimposed on the map. (a) The fit of the inhibitor to the density is illustrated, with a few protein side chains included to orient the viewer. Two electron density contour levels are displayed: The thin lines show the 3σ contour while the thick lines show the 14σ contour. An F_o(iodosuprofen) − F_o(flurbiprofen) difference map calculated with MIR phases from the flurbiprofen structure shows the same very strong density for the iodine atom, but no density for the light atoms, as is expected from the structural similarities between flurbiprofen and iodosuprofen (not shown). The iodine atom of the inhibitor is seen at the center of the 14σ density peak. The carboxylate group of the inhibitor engages in a salt bridge with Arg-120 and lies within hydrogen-bonding distance of the hydroxyl of Tyr-355. Glu-524 is also engaged in a salt bridge interaction with Arg-120. The iodothiophene moiety packs directly under Tyr-385. In this view, Ser-530 lies behind the inhibitor; its side chain hydroxyl is positioned to make a hydrogen bond with the ketone oxygen of the inhibitor. (b) Stereoview of the bound inhibitor showing the protein backbone and side chains making up the active site cavity. A portion of the heme group is seen at the top of the figure. Only the 14σ contour is shown for clarity. This view is related to part a above by a 90° rotation about an axis running vertically in the plane of the page. This figure and Figures 3 and 6 were made with the program SETOR (Evans, 1993).

is the same as that proposed for (*S*)-flurbiprofen (Picot et al., 1994). The α-methyl group of the inhibitor's propionic acid is tucked into a small hydrophobic cleft lined by leucine and valine residues. The *R*-stereoisomer would place the α-methyl not in this small pocket but in unfavorably close contact with Tyr-355 (see Figure 2a). A recent study (Bhattacharyya et al., 1996) showed that the mutation of Tyr-355 to phenylalanine substantially reduces the enzyme's stereoselectivity toward 2-methyl-arylpropionic acid drugs.

The two aromatic rings of the inhibitor extend upward from the propionic acid into the hydrophobic upper portion of the cavity. The planar thienyl group is twisted with respect to the phenyl ring, with a dihedral angle between the phenyl ring and thecarbonyl group of 49°; a similar, albeit less pronounced, torsion is seen in the crystal structure of suprofen (Peeters et al., 1983). The phenyl ring makes van der Waals interactions with a number of hydrophobic side chains lining the channel, including leucine, valine, and alanine side chains. The thiophene ring makes similar but less extensive interactions. The iodine atom of the inhibitor lies in a small pocket at the top of the channel and is in close contact with various hydrophobic side chains, including the aromatic ring of Tyr-385. In addition, the iodine lies 3.2 Å away from the backbone carbonyl oxygen of Leu-384, a distance smaller than the sum of the van der Waals radii (Nyburg & Faerman, 1985). While this distance cannot

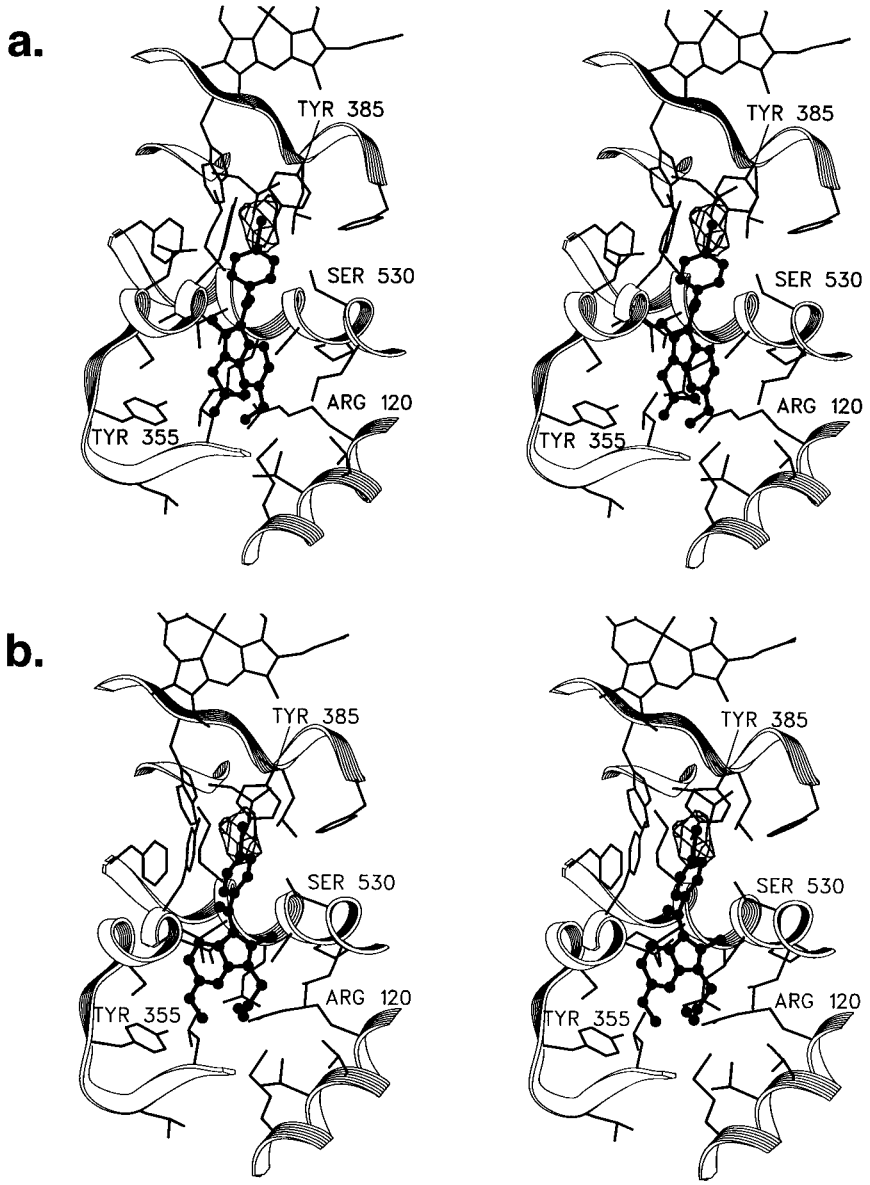


FIGURE 3: Stereoview of the difference electron density obtained for the iodoindomethacin molecule. This view is observed from essentially the same vantage point as Figure 2b. Shown is an $F_o - F_c$ map calculated after rigid body refinement of the starting model (see Experimental Procedures). Identical 10σ peaks corresponding to the two noncrystallographically related iodine molecules in the asymmetric unit were observed. No other peaks higher than 3σ were observed elsewhere in the map. A difference map (not shown) having coefficients of the form F_o(iodoindomethacin) $- F_o$(flurbiprofen) and using the MIR phases from the flurbiprofen model was essentially identical to the map seen here. (a) $F_o - F_c$ map contoured at 6σ. The refined position of the *cis* model of the iodoindomethacin molecule is shown, as are parts of the protein backbone and protein side chains lining the cyclooxygenase active. (b) As in (a) above, save with the *trans* model.

be considered extremely precise, given the resolution of the structure, it suggests that the iodine and oxygen atoms may be engaged in an interaction of the Lewis acid−Lewis base type, such as has been seen in small molecule crystal structures of iodine-containing organic compounds.

Iodoindomethacin−PGHS Complex Structure. As is the case with iodosuprofen, the iodine atom of iodoindomethacin is observed to bind at the top of the cyclooxygenase channel, packing directly against the aromatic ring of Tyr-385 (see Figure 5). This is the same site that is occupied by the iodine atom in the iodosuprofen structure; the distance between the iodine positions in the two structures is 0.6 Å. Placement of the iodine atom in the initial $F_o - F_c$ map was unambiguous (Figure 3). Density was also seen in this map for the light atoms of the iodoindomethacin molecule, but the quality of the map did not allow these atoms to be unambiguously fit into density. However, since the iodine lies at the very top of a relatively narrow channel, the possible orientations available to the remainder of the molecule are in fact quite limited. In addition, there are only five conformationally significant bonds in the inhibitor about which free rotation should be possible, and so the conformational space available to the molecule is not large. Hence, by fixing the iodine position and by assuming that the protein structure is the same as that found in the PGHS−flurbiprofen complex, it was possible to manually build in the light atoms of the iodoindomethacin molecule, using the X-ray crystal structure of the inhibitor as a guide (Loll et al., 1995b).

Steric constraints contributed by the protein side chains lining the channel severely limited the positions that the inhibitor atoms could occupy, and only two possible binding modes were identified. These modes correspond to the *cis* and *trans* conformers of the inhibitor, which are distinguished by a 180° rotation about the amide bond (see Figure 4).

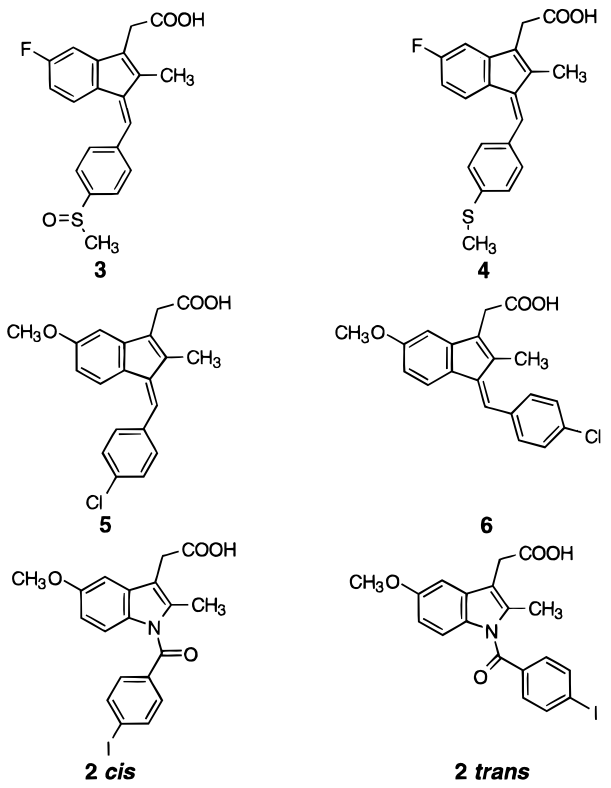


FIGURE 4: Indomethacin isosteres. **3** = sulindac; **4** = sulindac sulfide; **5** = (*Z*)-1-(*p*-chlorobenzylidene)-2-methyl-5-methoxyindenylacetic acid; **6** = (*E*)-1-(*p*-chlorobenzylidene)-2-methyl-5-methoxyindenylacetic acid. *Cis* and *trans* conformers of iodoindomethacin (**2**) are also shown.

Models of the inhibitor in both of these binding modes were constructed. Both agreed well with the density in the $F_o - F_c$ map, utilized reasonable values for the torsion angles of the inhibitor, and avoided serious steric clashes with the protein. These models were then subjected to limited refinement, which relieved unfavorable contacts and significantly improved the *R*-value without drastically changing the position of either the inhibitor or any active site side chains. The resulting models are chemically reasonable and agree well with the diffraction data. Given these facts and given the powerful constraints imposed by the inhibitor's position at the end of the narrow channel, it appears likely that one of the two models is correct. However, we cannot rigorously rule out the possibility that other binding modes may exist which we have failed to consider yet which are consistent with the observed iodine position. Hence, save for the iodine position, which is experimentally well determined, these models should be regarded as tentative.

In both our models for iodoindomethacin binding, the iodophenyl group of the inhibitor lies in a hydrophobic pocket at the top of the channel, with the iodine occupying the same position as that described for iodosuprofen above (see Figure 3). The carbonyl bridge between the phenyl and indole rings is situated further down the channel below the phenyl ring and is within 4 Å of several potential hydrogen bond donors, including the γ-hydroxyl of Ser-530. It is possible to bring the Ser-530 side chain into position to make a hydrogen bond with this carbonyl by a series of small torsion angle adjustments, indicating that the interaction is a feasible one. The indole rings in the *cis* and *trans* models overlap and are roughly coplanar; in both models the indole is in contact with several aliphatic side chains, including Val-349, Leu-359, Ala-527, and Leu-531. Both models predict a salt bridge interaction between the inhibitor's acetic acid moiety and the side chain of Arg-120, but these interactions differ significantly between the two models. In the *trans* model the acetic acid group is located between Arg-120 and Tyr-355 and could potentially participate in both a charged pair interaction with Arg-120 and in a hydrogen bond with Tyr-355. Dual interactions such as these are also seen with flurbiprofen (Picot et al., 1994), iodosuprofen, and salicylic acid (Loll et al., 1995a) in their respective complexes with PGHS. In contrast, in the *cis* model only Arg-120 makes an interaction with the inhibitor's carboxylate group, and Tyr-355 appears to be able to form a hydrogen bond with the ester oxygen of the inhibitor's methoxy group.

DISCUSSION

Use of Heavy-Atom Labeled Inhibitors. Crystallographic studies of drug−protein interactions depend critically upon the availability of well-diffracting crystals of the particular drug−protein complexes. In cases where data are available to very high resolution, the refinements are overdetermined and it is possible to obtain extremely precise models of the complex. However, for many important macromolecules, the best available crystals diffract X-rays to considerably more modest resolution. In such cases, the use of ligands labeled with electron-dense heteroatoms has historically proven immensely useful [see, for example, Olsen et al. (1976)]. Once the structure of the protein is known, low-resolution structures of heavy-atom labeled drugs complexed with the protein can be used in conjunction with modeling techniques to provide information about the interactions between the labeled compound and its protein target. While this information cannot approach the precision afforded by a high-resolution crystal structure determination, it should be emphasized that the heavy atom can be located with high accuracy. The heavy-atom reference point provides a powerful constraint which, when taken in conjunction with the steric constraints supplied by the active site pocket, limits possible binding modes for the compound of interest to a very small number.

Modeling of Iodoindomethacin Binding. We have identified two possible models of the PGHS−iodoindomethacin complex, starting from the experimentally determined iodine position. On the basis of the diffraction data we cannot discriminate between the *cis* and *trans* models; after refinement, their *R*-values and protein stereochemistry are of equal quality. In addition, there is little to suggest that one conformer is inherently more favorable than the other, since both conformations are observed in small molecule crystal structures (Kistenmacher & Marsh, 1972; Loll et al., 1995b), and molecular modeling calculations yield similar energies for both conformers (Loll, unpublished). We therefore attempted to discriminate between the two models using other criteria, including the models' agreement with structure−activity data for indomethacin analogs and the geometry of the important interaction between Arg-120 and the drug's carboxylate group.

Both models are in good agreement with the structure−activity profile of sulindac (**3**), a drug isosteric with indomethacin (see Figure 4). Sulindac is a prodrug which contains a sulfoxide moiety in a position analogous to those

occupied by the halogens in indomethacin and iodoindomethacin. The drug is inactive as the sulfoxide but becomes active upon metabolism to the sulfide **4** (Shen, 1979). Both of our models place the iodine atom of iodoindomethacin in a hydrophobic pocket directly under Tyr-385; we predict that the branched sulfoxide group of **3**, which is significantly larger and more polar than the iodine, would not be accommodated in this space. On the other hand, the less polar sulfide is only slightly larger than the iodine and should be accommodated.

We next consider structure−activity data for 1-benzylidenyindene isomers **5** (also known as MK-715) and **6**. The *Z*-isomer **5** is a five times more potent cyclooxygenase inhibitor than the *E*-form **6** (Hoogsteen & Trenner, 1970). This observation has been used to argue that the *cis* form of indomethacin, which is isosteric with the (*Z*)-indene, is the bioactive conformation (Gund & Shen, 1977). However, a 5-fold difference in affinity is not large, considering the large structural difference between **5** and **6**.

In studies of arginine−carboxylate interactions in crystal structures, clear preferences have been found for low (<45°) guanidinium−carboxylate interplanar angles and for the carboxylate oxygen accepting the hydrogen bond to lie in the plane of the guanidinium (Singh et al., 1987). In the *cis* model, the guanidinium−carboxylate angle is roughly 35°, and one of the carboxylate oxygens lies very close to the guanidinium plane; in the *trans* model the guanidinium−carboxylate angle is roughly 70°, and the oxygens both lie far from the guanidinium plane. Hence, geometrical arguments favor the *cis* model over the *trans*.

In conclusion, the relative affinities of **5** and **6** and the superior geometry of the carboxylate−arginine salt bridge in the *cis* model lead us to favor the *cis* model over the *trans*. However, we cannot rule out the possibility that both the *cis* and *trans* conformations of indomethacin bind PGHS; this would be consistent with the fact that **6** still inhibits the enzyme quite efficiently, even though it is 5-fold less potent than **5**. Heterogeneity of binding might also explain our inability to grow well-ordered crystals of the PGHS−indomethacin and PGHS−iodoindomethacin complexes. Thus, more experimental evidence will be required to completely elucidate the details of how drugs like indomethacin and sulindac inhibit PGHS.

Comparison of Different Modes of Inhibitor Binding. The NSAIDs display an unusually large degree of structural diversity. At least five structural classes of NSAIDs can be identified, with compounds from different classes bearing little or no resemblance to one another (Mantri & Witiak, 1994). This suggests the existence of distinct inhibitor binding sites or subsites on the enzyme which are used by the different inhibitor types. Alternatively, these inhibitors may employ different features of a common site for binding. To date, structures have been reported for complexes of PGHS with inhibitors belonging to three distinct classes: the salicylate 2-(bromoacetoxy)benzoic acid, an aspirin analog (bromoaspirin; Loll et al., 1995a), iodoindomethacin, an arylacetic acid, and flurbiprofen and iodosuprofen, both arylpropionic acids (Picot et al., 1994). These compounds differ widely in size and shape and inhibit PGHS by both covalent and noncovalent mechanisms. Hence, it is interesting to note that despite their structural and mechanistic differences, these inhibitors all share a common site of action (Figure 5).

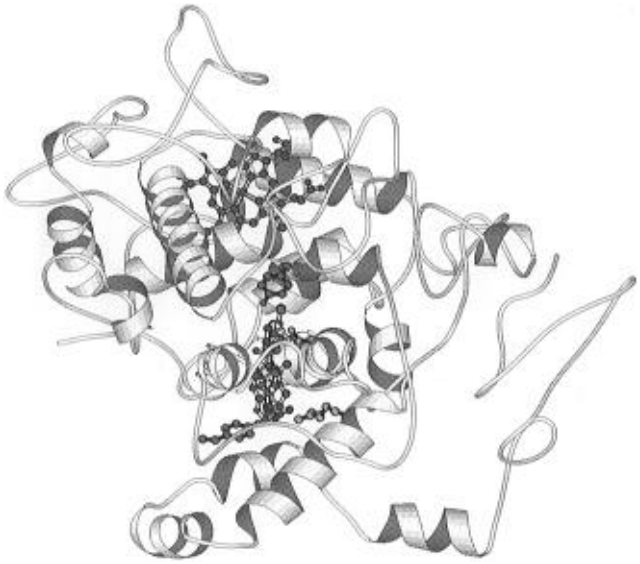


FIGURE 5: Demonstration of the marked overlap between the various inhibitor binding sites. Representation of the protein backbone of an entire PGHS monomer, viewed from the dimer interface. This is viewed from essentially the same direction as in Figures 2b and 3. The helices comprising the membrane binding domain are seen at the bottom of the figure, while the EGF-like domain is seen at the right. The heme is shown in red near the top of the molecule. Shown superimposed are the four inhibitors flurbiprofen (blue), iodosuprofen (green), the *cis* model of iodoindomethacin (red), and acetylated Ser-530 and salicylic acid (yellow). It can be seen that there is substantial overlap between the volumes occupied by the four inhibitors. Four protein side chains surrounding the inhibitors are shown: Tyr-385 (purple; above the inhibitor), Tyr-355 (purple; below the inhibitor on the left), Ser-530 (cyan; above and to the right of the inhibitors), and Arg-120 (cyan; below the inhibitor on the right). This figure was prepared with the program MOLSCRIPT (Kraulis, 1991).

The NSAIDs we have studied in complex with PGHS-1 all bind at the top of the channel leading from the exterior of the protein to the cyclooxygenase site, in close proximity to the putative catalytic residue Tyr-385. Their binding therefore blocks access of substrate to the active site cavity that lies at the end of this channel. The blockage is achieved in different ways by different compounds. For example, the acetyl group introduced by bromoaspirin lies near the top of the channel, just below Tyr-385; it does not fill the channel but evidently occupies enough volume to prevent the substrate from contacting Tyr-385. On the other hand, flurbiprofen, iodosuprofen, and iodoindomethacin are substantially larger than this acetyl group and fill the entire channel from Tyr-385 downward a distance of roughly 12−14 Å. Certain structural details are common to the binding of the different compounds. The carboxylic acid moieties of flurbiprofen, salicylic acid, iodosuprofen, and iodoindomethacin all form salt bridges with Arg-120, and at least some appear to form hydrogen bonds to the hydroxyl of Tyr-355. Since many of the different classes of NSAIDs carry carboxylic acid groups, these interactions may prove to be general features of binding for these drugs. This observation also suggests that the substrate arachidonic acid may bind with its carboxylic acid group making similar contacts with Arg-120 and Tyr-355.

Our observations also support the conclusion that a wide variety of ligands could occupy the cyclooxygenase catalytic pocket without obvious steric hindrance and without major conformational change in the protein structure between

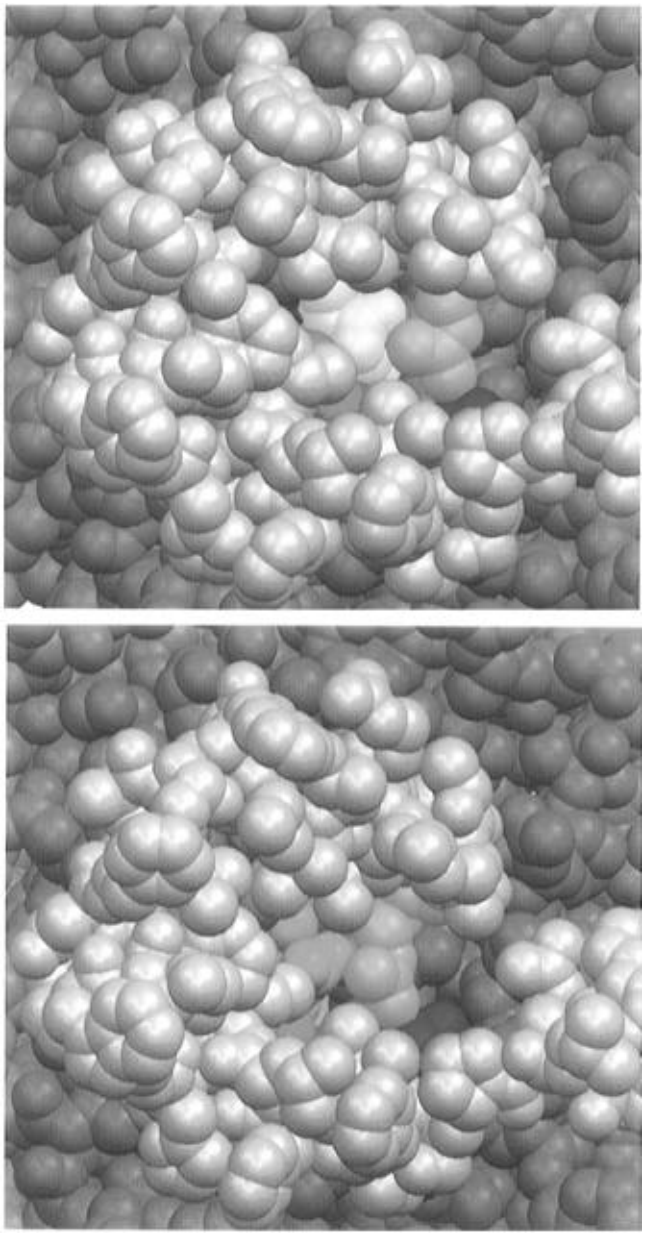


FIGURE 6: Space-filling models of the mouth of the cyclooxygenase channel viewed from the membrane. In (a, top) and (b, bottom), the catalytic domain is shown in dark gray while the amphipathic membrane binding domain is shown in light gray. Arg-120 (green) and Tyr-355 (blue) form part of the aperture; Glu-524 is shown in red. (a) Iodosuprofen (yellow) is shown bound within the channel, beyond the aperture; in (b) iodoindomethacin (orange) is shown bound. The membrane binding domain and the residues Arg-120 and Tyr-355 block the access of the bound drugs to the exterior of the enzyme toward the membrane side. Ligand binding and dissociation should involve structural alterations of the mouth of the cyclooxygenase channel.

different enzyme−drug complexes. The binding modes we have observed for the NSAIDs also clearly show that the NSAIDs occupy a site that is quite buried within the interior of the protein. As suggested by Loll et al. (1995a), several residues, including Tyr-355 and Arg-120, create an aperture or constriction between the channel mouth and the catalytic pocket. Space-filling models showing the mouth of the cyclooxygenase channel (Figure 6) reveal the extent to which the protein surrounds both iodosuprofen and iodoindomethacin. The buried nature of the bound NSAIDs strongly suggests that the drugs must effect a conformational change upon binding and subsequent dissociation. Such a structural change will surely involve conformational alterations of the aperture residues and, perhaps, the membrane binding domain. The magnitude of these conformational transitions or whether they can be correlated to the binding efficiencies of different NSAIDs is not known. Crystallographic analysis of the uninhibited holo-PGHS-1 may shed some light on the nature of these putative conformational changes. Although well-ordered crystals of uninhibited holo-PGHS-1 are more problematic to grow and handle, work toward this goal is underway.

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