Cyclooxygenase enzymes (COX-1 and COX-2) catalyze the conversion of arachidonic acid to prostaglandin G2. The inhibitory activity of rapid, reversible COX inhibitors (ibuprofen, naproxen, mfenamic acid, and lumiracoxib) demonstrated a significant increase in potency and time dependence of inhibition against double tryptophan murine COX-2 mutants at the 89/90 and 89/119 positions. In contrast, the slow, time-dependent COX inhibitors (diclofenac, indomethacin, and flurbiprofen) were unaffected by those mutations. Further mutagenesis studies suggested that mutation at position 89 was principally responsible for the changes in inhibitory potency of rapid, reversible inhibitors, whereas mutation at position 90 may exert some effect on the potency of COX-2-selective diarylheptocycle inhibitors; no effect was observed with mutation at position 119. Several crystal structures with or without NSAIDs indicated that placement of a bulky residue at position 89 caused a closure of a gap at the lobby, and alteration of histidine to typtophan at position 90 changed the electrostatic profile of the side pocket of COX-2. Thus, these two residues, especially Val-89 at the lobby region, are crucial for the entrance and exit of some NSAIDs from the COX active site.

COX-1 and COX-2 are sequence homodimers, but conformational heterodimers, with an allosteric monomer and a catalytic monomer. The monomers appear to be functionally interdependent, and data have shown that binding of a substrate or inhibitor at one active site alters the properties of the other active site (1, 2). A region along the dimer interface of the COX enzymes was suggested to allow for communication between the two COX monomers (3, 4). Non-steroidal anti-inflammatory drugs (NSAIDs) and other COX inhibitors act at both the allosteric and catalytic sites to modulate the activity of the COX isozymes (5–7).

Rome and Lands (8) were the first to report that some COX inhibitors display rapid, reversible inhibition, whereas others exhibit slow, tight binding inhibition. Ibuprofen and mfenamic acid are examples of inhibitors that display weak and rapid reversible inhibition of AA oxygenation, whereas flurbiprofen, despite its structural similarity to ibuprofen, exhibits slow, tight binding inhibition of the enzyme (9). Once bound, slow, tight binding inhibitors, which also include indomethacin and diclofenac, exhibit very slow off-rates from the enzyme, and are thus poorly reversible even in the presence of saturating concentrations of AA. In addition, COX-2-selective diarylheptocycle inhibitors (COXIBs), such as rofecoxib and celecoxib, demonstrate a second time-dependent step that is responsible for their isoform selectivity (10–12). X-ray crystallographic analysis and site-directed mutagenesis have been very useful in defining critical interactions between inhibitors and residues in the active site of the COX enzymes (13–15).

To date, the entrance/exit route(s) for inhibitors, substrates, and products of COX has not been well defined. Structurally, each monomer of COX-1 and COX-2 consists of an N-terminal EGF-like domain, a membrane binding domain (MBD), and a relatively large catalytic domain, which contains two independent active sites, the peroxidase (POX) site and the COX site.
COX inhibitors are thought to first enter through the four-helical MBD into an open area termed the “lobby” (the route is indicated in Fig. 1A) (13). The lobby is separated from the active site by three conserved residues, Arg-120, Tyr-355, and Glu-524, that act as a gate from the lobby to the active site. Another possible route, which lies between the opening above helix B of the MBD and the side pocket of the active site, has been proposed as an entrance and exit path for COX inhibitors to the active site (16). However, this route is much less likely, as it is too narrow for most NSAIDs. In the attempt to study the possible entrance/exit routes of different COX inhibitors, we created a series of double tryptophan mutants in the conserved locations in the MBD. The first mutant, V89W/S119W, was designed to reduce space in the lobby between helices B and D, mainly disrupting the pathway through the gate into the active site from the lobby. The second mutant, V89W/H90W, reduced space adjacent to and above helix B, constricting both the route through the lobby and the proposed alternate route (Fig. 1B). Evaluation of the impact of these mutations on a range of structurally and functionally diverse inhibitors revealed that the increased stericbulk in the lobby transformed rapid, reversible inhibitors into slow, tight binding inhibitors. The analysis of single tryptophan mutants revealed that the mutation primarily responsible for these mechanistic changes was V89W. Structural analysis by x-ray crystallography of these mutants indicated that tryptophan mutation at position 89 effectively reshapes the profile of the lobby route channel of COX-2, indicating that at least some inhibitors of COX enter/exit the active site mainly through this channel.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of COX-2 MBD Mutants**—Site-directed mutagenesis was performed on a pvL-1393 plasmid bearing the cDNA of murine COX-2 as previously described (15) to generate two double tryptophan mutants (V89W/S119W and V89W/H90W) and single tryptophan mutants (V89W, H90W, and S119W). The resulting mutants were expressed in Sf-21 insect cells and purified by sequential ion-exchange and size exclusion chromatography to >95% purity. The specific COX and POX activities of the mutants are characterized in Table 1.

**Oxygen Consumption Assays**—To determine COX activity, purified wild type and mutant mCOX-2 proteins (100 nM) were reconstituted with 2 eq of hematin and then incubated in a 1-ml thermostatted cuvette at 37 °C in a solution containing 100 mM Tris HCl, pH 8.0, and 0.5 mM phenol. AA was added to a final concentration of 50 μM, and oxygen consumption was monitored using a Hansatech OXYG1 plus connected to a DW1 oxygen electrode chamber controlled with Oxyview software (PP Systems Inc.). The reported rates of oxygen consumption were the maximal rates occurring after a lag phase. The lag time is defined as the time required to reach the maximal reaction velocity after the addition of AA.

**POX Activity Assays**—All reactions were carried out at room temperature. Wild-type and mutant mCOX-2 proteins (100 nM) were reconstituted by the addition of 0.5 eq of hematin and incubated for 1 min at room temperature in 100 mM Tris-Cl, pH 8.0, and 1 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Hydrogen peroxide was added to a final concentration of 50 μM. POX activity was monitored by the oxidation of ABTS to ABTS+ at 417 nm. The initial rate was calculated from the linear portion of the curve.

**Km and kcat Determinations**—50 nM wild-type or mutant mCOX-2 was incubated in enzyme assay buffer containing 100 mM Tris, pH 8.0, and 0.5 mM phenol at 37 °C for 3 min. After the preincubation, varied concentrations of AA (0.5, 1, 2.5, 5, 10, 25, 50, 75 μM) were added for 10 s at 37 °C. The reaction was quenched by the addition of ice-cold ethyl acetate containing 0.5% (v/v) acetic acid and 300 pm concentrations of internal standards (PGE2-d4). The solution was then vigorously mixed and cooled on ice, and the separated organic fraction was dried.
under nitrogen for the analysis of PGs by LC-MS/MS (17, 18). The kinetic data were fit using Prism Software to the Michaelis-Menten kinetic model.

**COX-2 Inhibition Assay—Time-dependent inhibition reactions** were run with purified, hematin-reconstituted native or mutant proteins. After preincubation of enzyme with inhibitors for the specified times, 50 μM [1-14C]AA was added to initiate the enzymatic reaction, which was allowed to proceed for 30 s at 37 °C. Reactions were then terminated and analyzed for substrate consumption by thin-layer chromatography as previously described (14).

For concentration-dependent inhibition reactions, preincubation of the desired concentrations of the inhibitors with protein for 17 min at 25 °C followed by 3 min at 37 °C preceded the addition of 50 μM [1-14C]AA. Alternatively, inhibitors were prebound to either wild-type mCOX-2 enzyme or the V89W/H90W mutant for 17 min at 25 °C followed by 3 min at 37 °C, and then [1-14C]AA at different concentrations (6–50 μM) was added, and reactions were allowed to proceed for 30 s. In all cases the final concentrations of the native or mutant proteins were adjusted to give a maximal 30–35% consumption of AA, whereas most of the measurements were conducted with a lower consumption of <10%. IC50 values were based on at least two independent determinations, and the values were calculated using Prism 6 software.

**Crystallization of mCOX-2 and Mutants—Crystallization** was performed as previously described with modest modifications (17, 18). Limited trypsin digestion has been used in the successful crystallization of COX-2 to remove the disordered C-terminal tail, which is not resolved in most crystal structures (15). In our hands, trypsin-treated protein generally crystallizes more readily than untreated protein. Thus 10 mg/ml purified trypsin-treated protein was used for crystallization. The holoenzyme was made by reconstitution with a 2-fold molar excess of Fe3+-protoporphyrin IX. Both apo- and holoprotein samples were dialyzed against 20 mM sodium phosphate buffer, pH 6.7, 100 mM NaCl, 0.01% NaN3, 0.6% (w/v) n-octyl-β-D-glucoside (β-OG) overnight, and the concentration of β-OG was then adjusted to 1.2% for crystallization. For mCOX-2 complexed with inhibitors, a 10-fold molar excess of the inhibitor from a 25 mM DMSO stock was added to protein samples. This protein-inhibitor complex solution containing about 5.6% (w/v) DMSO was incubated on ice for 20–30 min before crystallization. Crystallization was conducted using the hanging drop vapor diffusion method by mixing equal volumes of protein (or protein-inhibitor complex, typically 3.5 μl) with reservoir solution. Crystals were mounted after a couple of weeks of growth and flash-frozen in liquid nitrogen for shipment and data collection. Both apo- and holoenzyme complexes were tested for crystallization in each case. The presence or absence of heme had no significant effect on the ability to obtain satisfactory crystals for structure determination nor did it affect the profile of the COX active site; however, subtle differences were found in the POX active site where the heme group resides (19, 20). For most complexes studied here, the apoenzyme provided the best overall diffraction data, and these are the data reported.

**X-ray Data Collection, Structure Determination, and Refinement—X-ray data** were collected using the synchrotron radiation x-ray source with 100 K liquid nitrogen streaming at beamline 24-ID-E of the Advance Photon Source at the Argonne National Laboratory. Diffraction data were collected and processed with HKL 2000 (21) or XDS (22). The space groups were determined as P21, 212, 222, I4122, or C2 in different data sets (see Table 4). Initial phases were determined by molecular replacement using a search model (PDB ID 3NT1, chain A) with Phaser (23) in the resolution ranges of 107.7–2.76 Å for the COX-2 structure, 45.3–2.78 Å for the V89W-lumiracoxib complex, 49.3–2.43 Å for apoH90W, 45.1–2.57 Å for the H90W-lumiracoxib complex, and 48.3–2.81 Å for the H90W-ibuprofen complex (see Table 4). The score of the top solutions for the COX-2 structure was RFZ = 10.2, TFZ = 16.7, LLG = 20367, resulting in an R-factor of 37.7. Neither the rotational nor the translational searches yielded a second unrelated peak. Initial phases of other structures were obtained similarly and gave essentially the same parameters (RFZ = 6.0, TFZ = 9.3, LLG = 9314, R-factor = 37.9 for V89W-lumiracoxib complex; RFZ = 10.0, TFZ = 14.2, LLG = 28771, R-factor = 38.1 for the H90W structure; RFZ = 11.6, TFZ = 9.1, LLG = 26089, R-factor = 35.3 for the H90W-lumiracoxib complex; RFZ = 5.4, TFZ = 10.8, LLG = 16406, R-factor = 28.2 for the H90W-ibuprofen complex). The phases were improved with several rounds of model building against working data sets (F > 1.34 σF) in COOT (24) and Phenix (25), whereas 3.0% reflections (R free set) were set aside for quality control. Global non-crystallographic symmetry (if present) was applied during the refinement. Water molecules were adding during the last cycles of refinement, and translation-libration-screw refinement was applied in the last cycle. The potential of phase bias was excluded by simulated annealing using Phenix (26). The values of the Ramachandran plot for the final refinement of the structure were obtained by the Phenix suite. Data collection and refinement statistics are reported in Table 4. Crystal structures from different space groups were all essentially the same as those of the known COX-2 structures with very subtle structural fluctuations. The atomic coordinates and structure factor have been deposited in the Protein Data Bank. Because the root mean square deviation of the main chain and side-chain atoms between the different monomers (if present) in all complexes are within the range of 0.15-0.30 Å, no significant structural differences are evident among the monomers in the asymmetric unit. Therefore, all illustrations were prepared using the coordinates of monomer A with PyMOL (Schrödinger, LLC).

**RESULTS**

**Double Tryptophan Mutants in the MBD Convert Rapid, Reversible Inhibitors to Slow, Tight Binding Inhibitors—Mutations** were made at positions 89, 90, and 119 in MBD helices B and D to generate the double mutants V89W/H90W and V89W/S119W. Mutants were expressed in SF-21 cells and purified using published procedures (5). Despite the restrictions to the entrance of the active site, both of the mutants were active enzymes. Steady state kinetic studies revealed decreases in Km, for both proteins along with similar reductions in kcat, resulting in only a small decrease in overall catalytic efficiency (kcat/Km).
preincubation period to detect time-dependent interactions. mutants (V89W/H90W and V89W/V119W) in an assay with a tor was assayed against wild-type enzyme and the double indomethacin, diclofenac, and the coxibs (Fig. 2). Each inhibi-
mefenamic acid, and 2) time-dependent inhibitors, such as
screening purposes from two different categories: 1) competi-
tive, reversible arylcarboxylic acids, such as ibuprofen and
lumiracoxib, did not show potent inhibition toward the wild-
type enzyme, failing in most cases to reach 50% inhibition at
concentrations up to 10 μM. In contrast, the time-dependent
inhibitors, such as indomethacin, flurbiprofen, and diclofenac,
showed potent inhibition of mCOX-2 at concentrations in the
nanomolar range. When the inhibitors were assayed against
the V89W/H90W and V89W/S119W double mutants using the
20-min preincubation method, ibuprofen and other weak,
competitive inhibitors demonstrated an increase in potency
(IC50 values 100–200 nM), with a corresponding drop in the
time-dependent, non-selective inhibitors did not show a similar
increase in potency against the double tryptophan mutants;
time-dependent, non-selective inhibitors did not show a similar
increase in potency against the double tryptophan mutants;
the effect was most pronounced for ibuprofen and
lumiracoxib, the latter of which is a particularly poor inhibitor
of wild-type mCOX-2 despite its COX-2 selectivity profile for
human over ovine cyclooxygenase-1 (20). Interestingly, the
time-dependent, non-selective inhibitors did not show a similar
increase in potency against the double tryptophan mutants;
IC50 values for these compounds were comparable to those
observed with the wild-type enzyme. In contrast, when the
COX-2-selective inhibitors were assayed against the two
double mutants, the IC50 values for inhibition of the V89W/H90W
mutant were 10-fold higher than that of wild-type mCOX-2 for
both celecoxib and rofecoxib, whereas the IC50 for inhibition of
the V89W/S119W double mutant was comparable with that of
the wild-type enzyme (Table 2).

We further examined the effects of ibuprofen on the V89W/
H90W mutant to determine whether this rapidly reversible,
competitive inhibitor of the wild-type enzyme was still revers-
ible and competitive with respect to AA in the mutant. When
different concentrations of ibuprofen and AA were added
simultaneously to V89W/H90W in the absence of a preincub-
ation, there was no observable inhibition of the tryptophan
mutant enzyme by ibuprofen as compared with a DMSO con-
trol (Fig. 4A). In contrast, when a 20–300-s preincubation was
included before the addition of 50 μM AA, ibuprofen exhibited
increased inhibition with longer preincubation time (Fig. 4B).
Similarly, assay of the V89W/H90W mutant after a 20–min pre-
incubation with either ibuprofen or mfenamic acid before
the addition of 6–50 μM AA revealed a relationship between
inhibitor concentration and remaining activity that is con-

TABLE 1

| Mutants        | POX activity | COX activity | Rate | Km | kcat | kcat/Km |
|----------------|--------------|--------------|------|----|------|---------|
|                | Rate a       | % of wild-type enzyme | 10^3 s⁻¹ | μM | s⁻¹ | |
| mCOX-2         | 0.81 ± 0.01  | 100          | 0.16 | 2.29 | 2.13 | 0.57 |
| V89W           | 0.62 ± 0.05  | 77           | 0.10 | 1.60 | 1.37 | 0.69 |
| H90W           | 0.98 ± 0.08  | 122          | 0.12 | 0.72 | 1.39 | 0.56 |
| S119W          | 0.72 ± 0.03  | 90           | 0.10 | 0.71 | 2.39 | 0.10 |
| V89W/H90W      | 0.55 ± 0.02  | 68           | 0.10 | 2.07 | 2.86 | 0.30 |
| V89W/S119W     | 1.7 ± 0.1    | 207          | 0.10 | 1.60 | 1.93 | 0.05 |

a The POX activity was monitored by the oxidation of ABTS to ABTS⁺ at 417 nm at room temperature using 100 nM recombinant wild-type protein or mutants.

b Km and kcat values were calculated by the initial rates of prostaglandins from LC-MS measurements at various concentrations.

Rapid, Reversible Arylcarboxylic Acid COX Inhibitors

Slow, Tight-Binding COX Inhibitors

FIGURE 2. COX inhibitors used in the present study.

(Table 1). By oxygen consumption assay under saturating con-
ditions, the double mutant enzymes exhibited COX activities
ranging from 81% to 85% that of the wild-type enzyme, whereas
the POX activity of the V89W/H90W mutant was reduced and
that of the V89W/S119W mutant was increased relative to that
of mCOX-2 (Table 1). The observed changes in COX activity
suggested that the double mutations altered both substrate
binding and catalysis, with the most striking changes observed
for the V89W/H90W mutant.

For the inhibition assay, COX inhibitors were chosen for
screening purposes from two different categories: 1) competi-
tive, reversible arylcarboxylic acids, such as ibuprofen and
mfenamic acid, and 2) time-dependent inhibitors, such as
indomethacin, diclofenac, and the coxibs (Fig. 2). Each inhibi-
tor was assayed against wild-type enzyme and the double
mutants (V89W/H90W and V89W/V119W) in an assay with a
preincubation period to detect time-dependent interactions.

As shown in Fig. 3 and Table 2, in assays using 50 μM sub-
strate, rapid competitive inhibitors, such as ibuprofen and

A Single Tryptophan Mutation at Position 89 Changes the Profile of Rapid, Reversible Inhibitors—To assess which of the tryptophan mutations conferred the increase in potency observed with ibuprofen and other competitive inhibitors, we expressed and purified each of the single point mutations at positions 89, 90, and 119. The single mutations resulted in only minor changes in substrate binding or turnover as indicated by their kinetic constants, and the purified proteins exhibited almost the same COX and POX activities as those of the wild-type recombinant enzyme under saturating conditions (Table 1). These findings suggest that substrate penetration is not hampered by a single tryptophan replacement at those positions. The non-selective, time-dependent inhibitors did not show any difference in potency from wild-type enzyme when screened against each of the single tryptophan mutants, as anticipated from the lack of a difference against the double mutants (Fig. 6B, Table 3). The single mutant, S119W, failed to confer the increase in potency observed with the competitive inhibitors against the V89W/S119W mutant. The IC_{50} values against S119W were similar to those against wild-type mCOX-2 (Fig. 6A and Table 3).

Assaying the single V89W and H90W mutants against the competitive inhibitor class indicated that the increase in potency observed with the double tryptophan mutants could predominantly be explained by the single mutation at position 89 and in some cases also at position 90 (Fig. 6A, Table 3). Thus, a tryptophan at either position is sufficient to increase the potency of inhibition. It is also noted that single mutations at positions 89 and 119 had no effect on the inhibition curves of celecoxib and rofecoxib, as both inhibitors showed potent inhibitory activity similar to that observed with the wild-type enzyme (Table 1 and Table 3).

Mutations of Tryptophan at Position 89 Effectively Closes the Gap between Helices B and D—We utilized x-ray crystallography to gain insights about how the MBD mutants alter the bind-

FIGURE 3. Inhibition curves for different NSAIDs against wild-type mCOX-2 and the double tryptophan mutants V89W/H90W and V89W/S119W. The enzymes were preincubated with the indicated inhibitors for 17 min at 25 °C and then an additional 3 min at 37 °C before the addition of 50 μM [14C]AA at 37 °C. The curves for ibuprofen, mefenamic acid, indomethacin, and flurbiprofen are illustrated in panels A, B, C, and D, respectively. The data points for wild-type enzyme are labeled as closed circles, those of V89W/H90W are closed squares; and the V89W/S119W data are shown in open squares.

TABLE 2

Inhibition of double tryptophan mutants by NSAIDs and COXIBs

All IC_{50} data were calculated from two-four independent experiments. For experiments with mCOX-2, concentrations of inhibitor were extended past 50 μM, and inhibitors that showed >80% COX activity remaining at 10 μM are indicated as having a relative IC_{50} that is > 10 μM.

| Inhibitor   | V89W/H90W | V89W/S119W | Wild-type |
|-------------|-----------|------------|-----------|
| Rapid, reversible       |           |            |           |
| Ibuprofen         | 103       | 182        | >10,000   |
| Mefenamic acid    | 184       | 117        | >10,000   |
| Naproxen          | 78        | 169        | 1,200     |
| Lumiracoxib       | 65        | 75         | >10,000   |
| Slow, tight binding, non-selective | | | |
| Indomethacin      | 270       | 247        | 127       |
| Diclofenac        | 48        | 61         | 35        |
| Flurbiprofen      | 88        | 104        | 108       |
| Slow, tight binding, selective | | | |
| Celecoxib         | 320       | 61         | 40        |
| Rofecoxib         | 1150      | 280        | 255       |

Insights into the Entrance/Exit Route of COX-2

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of certain NSAIDs. For the first time, we obtained a structure of apo uninhibited mCOX-2 at 2.8 Å and a complex of V89W-lumiracoxib at 2.5 Å (Table 4).

Comparing the V89W-lumiracoxib structure with the wild-type lumiracoxib complex (20), we can conclude that the alteration of valine to tryptophan at position 89 does not cause any global alteration in the folding of COX-2. The backbone of MBD helix B in the V89W mutant is minimally altered as compared with that of the native structure, whereas the bulky tryptophan side chain, which is oriented toward the surface of the protein facing the membrane lipid bilayer, forms a hydrophobic interaction with Tyr-115 (Fig. 7). A space-filling presentation revealed that the tryptophan filled the opening gap between MBD helices B and D, forming a full-closed donut ring for the gate instead of the half-closed C-shaped opening of the lobby in the native enzyme (Fig. 7, A and B), whereas the overall structure of the active site is essentially unaffected (Fig. 7C). This observed donut ring at the lobby of the COX active site suggested that the interaction between inhibitors and the residues along the lobby entrance/exit route is a crucial determinant of inhibition kinetics. In the V89W mutant, the transformation of the rapid reversible inhibitors to slow tight binding inhibitors is due to the reduced space at the opening and poor flexibility of the tryptophan side chain.

Despite the changes in the lobby region, the binding of lumiracoxib in the COX active site is virtually the same as that observed in the wild-type-lumiracoxib complex (Fig. 8A); the carboxylic group of lumiracoxib is hydrogen-bonded to two key residues at the catalytic apex, Tyr-385 and Ser-530, and a rotation of Leu-384 was also observed (20). No difference was found upon comparing this crystal structure to that of the wild-type enzyme complexed with lumiracoxib (Fig. 7C), suggesting that once the inhibitors pass through the lobby into the active site, the structural determinants for interaction between the inhibitors and the enzyme were the same in both proteins.

FIGURE 4. Time-dependent inhibition of the mCOX-2 V89W/H90W mutant by ibuprofen. A, inhibition of V89W/H90W by ibuprofen in the absence of preincubation. Different concentrations (cross, 0 μM; closed circle, 62.5 nM; open square, 250 nM; closed square, 1.0 μM; open diamond, 4.0 μM) of ibuprofen were preincubated with 50 nM V89W/H90W mutant for the indicated times (0–300 s) before the addition of 50 μM AA. B, inhibition of V89W/H90W by 250 nM ibuprofen with the different preincubation times. Ibuprofen (250 nM) was preincubated with 50 nM V89W/H90W mutant for the indicated times before the addition of 50 μM of AA. Preincubation was conducted at 37 °C for the indicated times.

FIGURE 5. Time-dependent inhibition of the V89W/H90W mutant by ibuprofen and mefenamic acid. The mutant proteins were preincubated with inhibitor for 17 min at 25 °C and then an additional 3 min at 37 °C at different concentrations before the addition of 6–50 μM AA. Inhibitor concentrations are designated as open diamonds (0 μM), closed squares (62.5 nM), open squares (250 nM), closed circles (1.0 μM), and crosses (4.0 μM).
The Profile of the COX-2 Side Pocket Is Structurally Changed in the H90W Mutant—To investigate the role of His-90 in the alteration of the potency of some NSAIDs, we obtained crystal structures of the unoccupied H90W mutant, the H90W-lumiracoxib complex, and the H90W-ibuprofen complex.

Among all three structures, the unoccupied H90W mutant is packed in a previously published space group, whereas the H90W-lumiracoxib and H90W-ibuprofen complexes belong to two different space groups, C2 and I4122, respectively. The C2 crystals appeared as square plates in a couple of weeks. It is noted that the P21212, I222 and C2 space groups are packed almost the same, with a very small angle of rotation between two dimers if we overlay the dimers from the asymmetric unit (from the symmetric mates for the I222 space group), whereas those crystals appeared identically as squared plates and/or rods. The new I4122 crystals emerged as bipyramidal octahedrons after several months of crystallization and are different from the other space groups mentioned above.

The mutant H90W was folded in the same manner as the wild-type protein, without structural perturbation with the exception of the titled mutation. In all three mutant structures, the B factor was relatively higher for Trp-90 than those of adjacent residues, suggesting that this residue is thermodynamically more mobile than other residues in the same region. The indole moiety of Trp-90 is oriented toward the protein surface, whereas the nitrogen atom of the indole ring hydrogen-bonds to one solvent molecule (Fig. 9). The side chain of Trp-90 forms hydrophobic contacts to a hydrophobic region of Arg-513 and Pro-514. An effective hydrophobic stacking of the aromatic ring to the n+4 residue (Thr-94) in MBD helix B is also present. In this orientation, the tryptophan...
does not significantly alter the structure of the lobby, and the overall integrity of the active site is retained (Fig. 7C).

The selectivity of celecoxib and rofecoxib for COX-2 arises from insertion of the sulfonamide and methyl sulfone moiety, respectively, into the COX-2 side pocket made up of His-90, Arg-513, Phe-518, Val-523, and Glu-524. The orientation of tryptophan in the H90W mutant exerts only minor changes to the COX-2 side pocket (Fig. 9); however, the electrostatic profile of the side pocket is altered. The thermodynamic instability of Trp-90 may explain why celecoxib and rofecoxib exhibited reduced potency in the H90W mutant. In addition, this displacement effectively lessens the flexibility of the region. These observations also help to explain why the H90W mutant promotes the transformation of rapid, reversible to slow tight binding inhibitors but does not do so as efficiently as the V89W mutant.

In both complexes of ibuprofen and lumiracoxib with the H90W mutant, the inhibitors were bound in the same way as in the respective wild-type complexes (Fig. 8B) (20, 27), indicating that the mutation at the lobby of the active site does not alter the binding modes of inhibitors but effectively causes changes in the dynamics of the binding process.

It should be noted that the slowly grown H90W-ibuprofen complex in the $I_{4}122$ space group resolved several C-terminal residues 584–589 that have not been seen in either COX-1 or COX-2. It is also noted that the heme cofactor in the same complex was poorly resolved in the crystal structure.

**DISCUSSION**

In the present studies we have structurally altered the membrane binding region of murine COX-2 by replacing several residues on helices B and D with bulky tryptophans in an effort to block the entrance/exit route(s) of inhibitors from the active site of the enzyme (Fig. 1). Careful examination of the wild-type COX-2 structure reveals that the lobby region is made exclusively of hydrophobic residues, including Val-89, Leu-93, Trp-100, Ile-112, Tyr-115, and Val-116 (Fig. 7). The MBD helices A–D form a pseudo-squared shape immediately beneath the exit of the active site. In the wild-type enzyme, this exit is half open to the side of helix A, where a gap is present between Val-89 and Tyr-115. Our x-ray structure of the V89W mutant suggests that this mutation at position 89 closes the gap in the MBD region.

When tested against the tryptophan mutants, the competitive inhibitor class demonstrated a significant increase in potency and extent of inhibition that was similar even among inhibitors of different structural scaffolds. Furthermore, ibuprofen exhibited time-dependent inhibition of V89W/H90W,
indicating that access of these inhibitors to the active site had been restricted. In contrast, tight binding inhibitors, such as indomethacin, were unaffected by these mutations, whereas COX-2-selective inhibitors exhibited a decrease in inhibitory activity against V89W/H90W compared with native enzyme. It is important to note that both sets of double mutants showed similar results. Thus, effectively blocking the lobby with tryptophans at positions 89 and 119 or blocking half of the lobby along with the area above helix B with tryptophans at positions 89 and 90 generated the same increase in potency observed with ibuprofen and the other competitive inhibitors. Results from single tryptophan mutant assays indicate that the V89W mutation is primarily responsible for the increased potency of competitive inhibitors against the tryptophan mutant enzymes. It is probable that the bulky tryptophan mutations on the MBD helices decrease the accessibility of the COX active site for the competitive inhibitors and hinder the departure of those inhibitors once they are bound to the active site.

Crystallographic analysis of the V89W mutant revealed that adding a bulky residue at helix B resulted in a closed donut-shaped opening at the base of the MBD compared with the half C-shaped opening of the native enzyme. Thus, in the inhibition assay with an adequate preincubation time, these rapid reversible inhibitors exhibited a distinct time-dependent inhibition that is similar to the profile of slow, tight binding inhibitors such as indomethacin. On the other hand, crystal structures of the H90W mutant suggested that the H90W substitution blocks the other proposed route between helix B and the side pocket for dissociation from the enzyme. The decrease in the potency of COX-2-selective inhibitors against the tryptophan mutants can largely be attributed to the H90W substitution. Even though the crystal structures of the H90W mutant disclosed no significant changes in the volume of the side pocket, a possible alternative orientation of Trp-90 was not excluded, as this residue is more mobile than the adjacent residues indicated by higher temperature factors. In addition, the mutation of His-90 to a bulky tryptophan residue could potentially alter the features of the COX-2 side pocket and hinder the flexibility of the MBD in such a way that coxibs can no longer efficiently bind in this area of the active site. This would lead to a decrease in the inhibitory activity of coxibs. Thus, the H90W mutant behaved dramatically differently from the native protein with regard to the COX-2-selective inhibitors.
In contrast to the rapid reversible inhibitors, slow, tight binding inhibitors displayed similar inhibitory activity against the wild-type and all of the tryptophan mutants. This observation is likely explained by the fact that the dissociation rate of the tight binding inhibitors is very slow and is, therefore, less likely to be significantly affected by steric hindrance at the entrance/exit routes introduced by tryptophan mutations. However, we cannot rule out the possibility that these inhibitors access the active site by an as yet unexplored route.

It is quite striking that the entrance of substrate AA and the exit of the PG product were not substantially hindered by the introduction of a single bulky tryptophan side chain, as indicated by the minimal effects of the single tryptophan mutations on $K_m$ and $k_{cat}$ values of COX-2 (Table 1). Notably, the catalytic efficiency of the V89W mutant is essentially the same as that of the wild-type enzyme despite the striking differences between the two proteins with regard to the potency of the rapidly reversible inhibitors. In contrast, the double mutants both show a decrease in $K_m$ and $k_{cat}$ values relative to those of mCOX-2. To the degree that $K_m$ is a reflection of the affinity of the enzyme for substrate, these changes are consistent with a tighter association of the substrate with the active site. Furthermore, the reduction in $k_{cat}$ might be attributable to a reduced rate of exit of the product resulting from the constriction of the channel.

Taken together, these studies demonstrate that one single mutation at the entrance/exit route of the lobby in COX-2, which is below the active site of the enzyme, is effective in altering the dynamics of inhibitor association and dissociation without altering the molecular interactions between the bound inhibitors and COX. Accumulating evidence suggests that binding of substrates or inhibitors within the COX active site can alter these interactions (3, 4), and the data presented suggest that structural changes in the MBD of COX enzymes also alter the dynamics of enzyme-inhibitor interactions by narrowing the accessible size of the channel. These tryptophan lobby mutants will be excellent tools to explore the binding of COX inhibitors to the COX active site for further kinetic and structural analyses.

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