Inhibitor-Induced Changes in the Intrinsic Fluorescence of Human Cyclooxygenase-2

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ABSTRACT: The steady state tryptophan fluorescence of apo-human cyclooxygenase-2 (hCox-2) is quenched approximately 40%−50% by the slow binding inhibitors diclofenac, indomethacin, ketoprofen, NS-398, and DuP-697. The effects of these inhibitors on tryptophan fluorescence are both time and concentration dependent. Addition of each inhibitor results in a rapid fluorescence decrease, followed by a slower time dependent quenching. The slow, time dependent loss of fluorescence follows first-order kinetics, the rate constants for the process increasing with inhibitor concentration in a saturation-type manner. The rapid fluorescence loss also increases with increasing inhibitor concentration in the same manner. These results are consistent with the initial formation of a rapid equilibrium complex of enzyme and inhibitor (EI), followed by the slower formation of a tightly bound enzyme−inhibitor complex (EI*). The fluorescence of the EI complex is not significantly different from that of the EI* complex. The kinetic parameters of each inhibitor derived for this process (K_i and k_{on}) are close to those obtained by determination of the rate constants for the onset of enzyme inhibition, thereby linking the fluorescence changes with inhibitor binding. The reversible inhibitors ibuprofen and docosahexaenoic acid do not quench the protein fluorescence but do decrease both the rate of the slow fluorescence loss and the magnitude of the initial rapid fluorescence decrease caused by the slow binding inhibitors, consistent with their competitive behavior. ASA-acetylated apo-hCox-2 shows the same fluorescence-quenching behavior in the presence of most of the above inhibitors. However, acetylation apparently blocks the binding of diclofenac, whereas the affinity of ibuprofen is increased. The effects of the collisional quenching agents iodide and acrylamide on both the native and inhibited enzyme are small (<20% quenching at 0.3 M), showing that inhibitor binding does not result in an increased solvent accessibility of protein tryptophans. The cause of the inhibitor-induced quenching of the intrinsic apo-hCox-2 fluorescence is likely energy transfer to the bound inhibitor. Calculations based on the inhibitor−tryptophan distances in ovine Cox-1 indicate that the distances are within the required range for significant quenching to occur.

Cyclooxygenase (Cox)[1] catalyzes the oxygenation of arachidonic acid to form the endoperoxide hydroperoxide PGG_2 (cyclooxygenase activity) and its subsequent reduction to the endoperoxide alcohol PGH_2 (peroxidase activity). Cell specific synthases reduce or isomerize PGH_2 to a number of biologically active prostanoids (Smith & Marnett, 1991, 1994; Marnett & Maddipati, 1990). Two isoforms of Cox have been identified. The constitutive form (Cox-1) is believed to be involved with the maintenance of normal cellular physiology, whereas the recently identified inducible form (Cox-2) produces prostanoids involved in inflammation, mitogenesis, and tumor progression (Goppelt-Struebe, 1995; Smith & DeWitt, 1995).

The primary sequences of the two human isoforms share 61% identity, and there is conservancy of the proposed catalytic residues, as well as structural domains and N-glycosylation sites (Hla & Nielson, 1992). The two isoforms are physically and functionally similar (Percival et al., 1994; Barnett et al., 1994; Gierse et al., 1995), although they do have different requirements for the reaction initiator hydroperoxide (Capdevila et al., 1995) and also can be distinguished on the basis of their response to inhibitors such as ASA (Holtzman et al., 1992; Lecomte et al., 1994), NS-398, and DuP-697 (Copeland et al., 1994; Ouellet & Percival, 1995; Gierse et al., 1995).

Cox-2 is the anti-inflammatory target of the therapeutic agents known as NSAIDs, but inhibition of Cox-1 likely results in their deleterious side effects (Monroe & Lau, 1995). Currently available NSAIDs do not show any appreciable selectivity for Cox-2 over Cox-1 (O'Neill et al., 1994; Meade et al., 1993). In general, NSAIDs inhibit only the cyclooxygenase activity, and in most cases, inhibition is competitive with arachidonic acid (Rome & Lands, 1975; Copeland et al., 1994). NSAIDs can be divided into three groups according to the mechanism of their inhibition of Cox. Compounds such as indomethacin, flurbiprofen, and diclofenac inhibit in a two-step, time dependent mechanism involving a rapid equilibrium binding of inhibitor followed by the slower isomerization to a more tightly bound complex. The formation of this complex is essentially irreversible, but it is non-covalent, since inhibitor can be recovered intact upon protein denaturation (Kulmacz & Lands, 1985; Copeland et al., 1994). The second slow step has been suggested

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[1] Abbreviations: o, ovine; h, human; Cox, cyclooxygenase; PG, prostaglandin; DHNBS, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; NSAID, non-steroidal anti-inflammatory drug; ASA, aspirin; 15-HETE, 15-hydroxyeicosatraenoic acid.

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to involve a protein conformational change (Kulmacz & Lands, 1985), although its nature remains presently unknown. Members of the second group, such as ibuprofen, act as simple reversible inhibitors (Rome & Lands, 1975; Smith et al., 1995). The single member of the third group is ASA, which causes the acetylation of a single serine residue, thereby interfering with arachidonic acid binding (Roth et al., 1983; Holtzman et al., 1992; Lecomte et al., 1994).

Inhibitor binding to oCox-1 can be monitored by the decreased protease sensitivity of the enzyme−inhibitor complex (Kulmacz, 1989). However, spectroscopic techniques such as circular dichroism (Kulmacz, 1989) and heme absorption (Kulmacz & Lands, 1985) have failed to detect any ligand-induced changes.

Here we report on the effects of NSAID binding on the steady state intrinsic protein fluorescence of apo-hCox-2. Most of the NSAIDs tested caused a quenching of protein fluorescence, and the kinetic parameters for inhibitor binding correlated well with those obtained from measurement of enzyme inhibition. The results are consistent with inhibition by time dependent inhibitors occurring via a two-step mechanism, although our results do not support the notion that the kinetically detectable isomerization of the initial enzyme−inhibitor complex (EI) to the tightly bound form (EI*) involves a major change in the inhibitor binding mode.

MATERIALS AND METHODS

Diclofenac, ketoprofen, and docosahexaenoic acid were from Sigma. NS-398 and DuP-697 were prepared by the Department of Medicinal Chemistry, Merck Frosst Canada. All other NSAIDs were from Cayman Chemical Co.

Recombinant hCox-2 was expressed in a baculovirus-Sf9 cell system and purified as previously described (Percival et al., 1994). The cyclooxygenase activity of hCox-2 was determined by oxygen uptake as described (Percival et al., 1994). The specific activity of holoenzyme was 29 μmol of O_2/min/mg. Protein concentration was determined by coomassie blue dye binding assay using bovine serum albumin as standard (BioRad).

In preparation for fluorescence studies, the enzyme was concentrated to approximately 1.5 mg/mL on a Centricon-30 device (Amicon) and then desalted by passage through a fast desalting column (Pharmacia) in buffer containing 20 mM MOPS, 0.1% β-octyl glucoside, pH 7.5. The final protein concentration was typically 0.25−0.4 mg/mL. The proportion of holoenzyme was determined from the protein absorbance at 408 nm using an extinction coefficient of 128 mM^{-1} cm^{-1} (Percival et al., 1994). Desalted hCox-2 routinely contained 10%−15% holoenzyme. This value was confirmed by measurement of the cyclooxygenase activity in the absence of heme in the assay buffer.

ASA-acetylated apo-hCox-2 was prepared by incubation (14 h) of enzyme (0.47 mg/mL) at 22 °C with 10 mM ASA or vehicle, added as a solution in 50% aqueous DMSO to a final concentration of 2%. The protein was desalted as described above, and an aliquot (0.3 μg) was assayed for 15-HETE synthase capacity as previously detailed (Cromlish et al., 1994) in the presence of 0.5 mg of stannous chloride/mL. The reaction time was 30 min. The reaction mixtures were applied directly to a silica gel TLC plate which after drying was developed with benzene/dioxane/formic acid/acetic acid [82:14:1:1 v/v, Lecomte et al. (1994)]. The products were quantitated by densitometry on a Berthold LB2842 TLC linear analyzer. ASA-treated hCox-2 synthesized 15-HETE and $PGF_{2\alpha}$ in a ratio of 2:1, whereas vehicle-treated hCox-2 synthesized $PGF_{2\alpha}$ exclusively. The oxygenase activities of ASA- and vehicle-treated hCox-2 were 7.2 and 18 μmol of O_2/min/mg, respectively. The above conditions for ASA-acetylation were employed because of the previously observed (Kulmacz, 1989) slow inhibition of ovine apo-Cox-1 by ASA as compared to the holoenzyme. Subsequent experiments showed that inhibition of apo-hCox-2 cyclooxygenase activity was maximal after 30 min in the presence of 10 mM ASA, but longer incubation periods did not lead to inactivation of 15-HETE synthase activity. This is in contrast to recent results (Laneuville et al., 1995) but may reflect differences between microsomal and cellular enzyme and purified apo-hCox-2, or the incubation temperature (37 °C versus 22 °C employed here).

Fluorescence measurements were performed on a Perkin Elmer LS-5 spectrometer under computer control. Excitation and emission monochromator slit widths were 2.5 and 10 nm, respectively, except for emission wavelength scans, in which case the emission slit width was 5 nm. Measurements were performed at 25 °C in 1 mL quartz cuvettes, and solutions were mixed continuously by a magnetic stirrer. Data points were collected at 0.1 or 1 s intervals during time courses. Corrections for the inner filter effect were performed where noted according to the formula (Lakowicz, 1983)

$$F_c = F \,\text{antilog}[(A_{ex} + A_{em})/2]$$

where F_c and F are the corrected and measured fluorescence intensities, respectively, and A_{ex} and A_{em} are the solution absorbances at the excitation and emission wavelengths, respectively. Background buffer spectra were subtracted to remove the contribution from Raman scattering. Fluorescence spectra were not corrected for the wavelength variation in detector response. Total emission spectra (tryptophan plus tyrosine) were measured by excitation at 280 nm. Emission spectra due to tyrosine were obtained by subtraction of the tryptophan emission spectra (excitation at 295 nm) from the total emission spectra after normalization of the former at 370 nm where tyrosine fluorescence is negligible (Weinberg & Jordan, 1990).

For inhibitor binding studies hCox-2 was diluted in 100 mM Tris, 5 mM EDTA, pH 8.0. The final protein concentration was typically 18 μg/mL (0.24 μM). At this protein concentration, the fluorescence of apo-hCox-2 was stable for up to 15 min, although lower concentrations (>5-fold) showed a gradual fluorescence decrease (6% min^{-1}) presumably due to denaturation or losses on the cuvette walls. Apo-hCox-2 was thermally denatured by heating a 18 μg/mL solution at 95 °C for 5 min in 100 mM Tris, 5 mM EDTA, pH 8.0. Heat-treated enzyme had no activity when assayed as previously described (Ouellet & Percival, 1995). Inhibitors (1.0 μL aliquots) were added from solutions in DMSO by Hamilton syringe. DMSO itself (0.1% final concentration) had no effect on the protein fluorescence. A new enzyme solution was used for each inhibitor concentration. Time courses of the protein fluorescence following inhibitor addition were measured for up to 10 min with excitation and emission wavelengths of 280 and 325 nm, respectively. The time required for complete mixing (>95%) of drug and

protein was estimated from the increased fluorescence of buffer alone on the addition of flurbiprofen in DMSO. Mixing was complete within 1 s.

The magnitude of the inhibitor-induced rapid decrease in fluorescence was determined from the change in fluorescence intensity within 1.0 s of addition of inhibitor. Small corrections (9% at the highest diclofenac concentration) were made for the inner filter effect as described above. Rate constants for the slow time dependent decrease in fluorescence (k_{obs}) at each inhibitor concentration were calculated by computer fitting of the remaining data, starting 1 s after inhibitor addition, to a first-order equation of the form $y = a + b \exp(-k_{obs}t)$ using Kaleidagraph software (Synergy Software). These values of k_{obs} were then computer fitted to the equation $k_{obs} = k_{on}[I]/(K_i + [I])$ to give calculated values of K_i and k_{on}. The K_i values of ibuprofen and docosahexaenoic acid were calculated by fitting k_{obs} values for the slow fluorescence decrease due to diclofenac (1.0 and 10.0 μM) at a range of concentrations of ibuprofen or docosahexaenoic acid (1.0−3.0 μM) to the equation (Kulmacz & Lands, 1985) $k_{obs} = k_{on}/(1 + (K_i/[I])(1 + [I']/K_i'))$. K_i' and I′ refer to ibuprofen or docosahexaenoic acid. The results from six k_{obs} determinations were averaged to calculate the K_i of ibuprofen, and four were used for docosahexaenoic acid. A single combination of the inhibitors NS-398 (5 μM) and ketoprofen (5 μM) was also used to determine the K_i value for ibuprofen (at 3 μM). The above equation was also used to estimate the K_i and k_{on} values of DuP-697. The value assumed for the K_i of ibuprofen was 0.2 μM. In some series of experiments a slower cuvette stirring rate was employed giving a mixing time (>95% complete) of less than 5 s. In these cases the $t_{1/2}$ for the slow fluorescence decrease was greater than 20 s, and therefore an insignificant amount of data was lost in the mixing time. For these experiments the rate constants for the slow decrease in fluorescence were fitted as described above but starting 5 s after inhibitor addition.

The determination of inhibitor K_i and k_{on} values for hCox-2 from the observed rate constants for the onset of inhibition was by a modification of a published procedure (Copeland et al., 1994). The buffer was 100 mM Tris, pH 8.0, 1 μM hematin, 1.0 mg of gelatin/mL. Inhibitors were added from a stock solution in DMSO to a final concentration of 1%. Following the prescribed preincubation period, the reaction was initiated by addition of 100 μM arachidonic acid and 100 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine in 50% aqueous ethanol (0.1 vol). Data were fitted as described above. Experiments to compare the rates of onset of inhibition of apo and holo-hCox-2 were performed as described above except that for apo-hCox-2 the incubation buffer did not contain hematin. For apoenzyme, hematin (10 μM) was added with the ethanolic substrates solution. The control (no inhibitor) reaction rates (150 mOD/min) were the same for apo- and holo-hCox-2.

The stoichiometry of diclofenac inhibition of hCox-2 was determined by preincubation of inhibitor (0−4 μM), added from a 50-fold concentrated stock solution in DMSO, with apo-hCox-2 (3.4 μM) in 100 mM Tris, pH 8.0, 5 mM EDTA at 22 °C. The cyclooxygenase activity remaining after 15 min of preincubation was determined by oxygen electrode (Percival et al., 1994) on a 20 μL aliquot. Longer preincubation times did not give higher levels of inhibition. The results were the average of duplicate determinations at eight diclofenac concentrations.

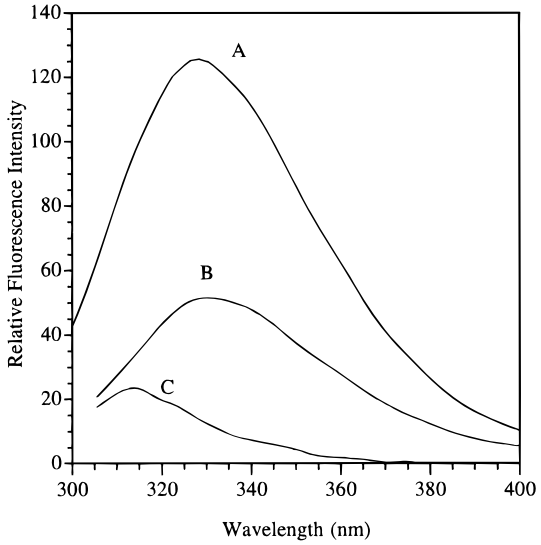

FIGURE 1: Steady state fluorescence emission spectra of apo-hCox-2. The spectra were measured with the following excitation wavelengths: (A) total fluorescence, 280 nm; (B) tryptophan fluorescence, 295 nm; (C) tyrosine fluorescence, 280 nm, determined as detailed in Materials and Methods. The protein concentration in all spectra was 18 μg/mL.

Titrations of native apo-hCox-2 and diclofenac-inhibited apo-hCox-2 with the collisional quenchers KI, CsCl, and acrylamide were performed by the sequential addition of a stock solution of each agent or KCl control. Corrections were made for the small dilution effect (2.5% for acrylamide and 7.5% for KI and CsCl for the highest concentrations used). For acrylamide, the resulting spectra were also corrected for the inner filter effect.

Chemical modification studies with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS) were performed at room temperature in 100 mM citrate, pH 6.0, with 1.0 mg of apo-hCox-2 or indomethacin-inhibited apo-hCox-2/mL. DHNBS (100 or 1000 molar excess) was added from a 25 mM aqueous solution, and the reaction was terminated after 10 min by passage through a gel filtration column as described above. The extent of modification was determined as previously described (Werber et al., 1987). Modifications under denaturing conditions were performed in the presence of 1% SDS.

Spectral overlap integrals, R_0 values, and relative quantum yields (Q_p/Q_a) were calculated using the Forster theory of fluorescence energy transfer (Cheung, 1991; Fox et al., 1993). The values of the constants used in the calculation of R_0 were from Fox et al. (1993).

RESULTS

Intrinsic Fluorescence of Apo-hCox-2. Apo-hCox-2 exhibits a typical steady state protein fluorescence with maximal excitation and emission wavelengths of 280 and 327.5 nm, respectively (Figure 1). The emission spectrum maximum and shape do not change on excitation from 260 to 280 nm, and excitation at 295 nm, where tyrosine has negligible absorption, results in an emission maximum of 331 nm (Figure 1). The emission due to tyrosine excitation (see Materials and Methods) contributes about 9% to the total fluorescence at 325 nm with excitation at 280 nm (Figure

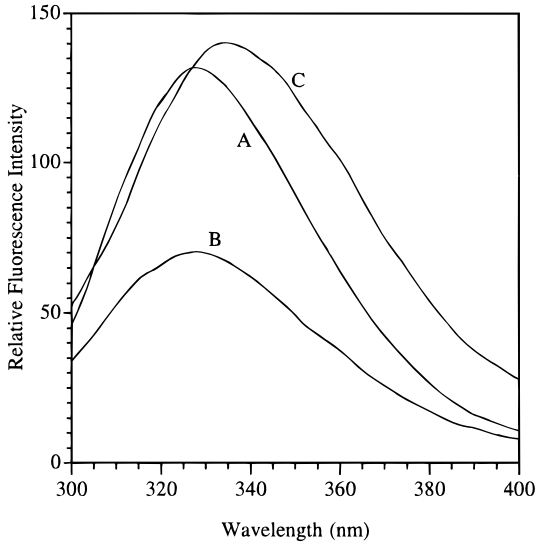

FIGURE 2: Effect of diclofenac on the fluorescence emission spectrum of apo-hCox-2. Native or heat-denatured apo-hCox-2 (18 μg/mL) was treated with diclofenac for 10 min as described in Materials and Methods. (A) Apo-hCox-2; (B) apo-hCox-2 treated with 1.0 μM diclofenac for 10 min; (C) heat-denatured apo-hCox-2. Addition of 1.0 μM diclofenac had no effect on spectrum C. The excitation wavelength was 280 nm in each case.

Table 1: Effect of NSAIDs on the Intrinsic Fluorescence of apo-hCox-2

inhibitor[a]	fluorescence intensity (%)	emission wavelength shift (nm)[b]
none	100	–
diclofenac	53	1.5
DuP-697	47	2.5
indomethacin	51	2.5
ketoprofen	62	1.5
NS-398	53	2.5

[a] apo-hCox-2 (18 μg/mL) was treated with 1.0 μM of each inhibitor. The excitation and emission wavelengths were 280 and 325 nm, respectively. [b] Shift from emission maximum of 331 nm with excitation at 295 nm for native apoprotein.

1). Tryptophan fluorescence typically shifts from an emission maximum of 350 nm in water to 310−324 nm in nonpolar protein regions (Lakowicz, 1983), suggesting that the majority of tryptophan fluorescence in apo-hCox-2 results from residues in relatively nonpolar environments.

Effect of Time Dependent Inhibitors on Apo-hCox-2 Fluorescence. When the Cox inhibitor diclofenac (1.0 μM) was added to apo-hCox-2, the total protein fluorescence intensity (excitation at 280 nm) was reduced by 47% (Figure 2, Table 1). The tryptophan emission spectrum (excitation at 295 nm) was shifted slightly from 331 to 332.5 nm, and the intensity was reduced by a percentage similar to that observed with total protein fluorescence. The tyrosine contribution to the total emission intensity at 327 nm increased from 9% to 14% in the presence of diclofenac, indicating that the intensity decrease is due mainly to quenching of tryptophan residues.

The tryptophan fluorescence maximum of heat-inactivated apo-hCox-2 was red shifted 7 nm (Figure 2), consistent with an increased solvent accessibility of those residues in the denatured protein. Treatment of this material with 1.0 μM diclofenac gave no change in the intrinsic protein fluorescence emission maximum or intensity, demonstrating that

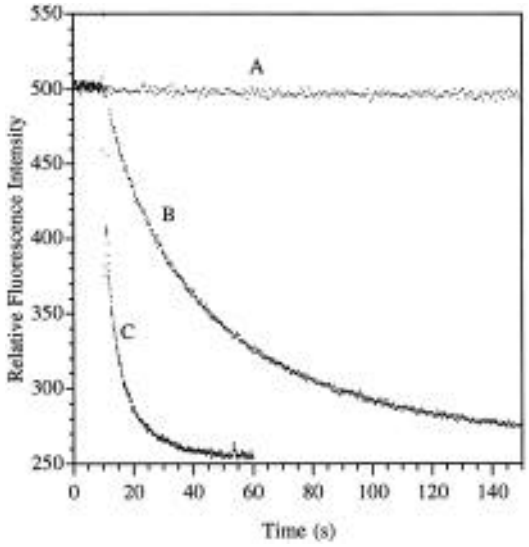

FIGURE 3: Time and concentration dependent effects of diclofenac on apo-hCox-2 fluorescence. Apo-hCox-2 (18 μg/mL) was treated at time 10 s with the following concentrations of diclofenac: (A) vehicle (DMSO); (B) 1.0 μM; (C) 10 μM, data after the addition of 10 μM diclofenac were multiplied by 1.05 to account for the inner filter effect. The excitation and emission wavelengths were 280 and 325 nm, respectively. The mixing time was 1 s, and the data interval was 0.1 s.

the above quenching is due to a specific interaction which is dependent on the native structure of the protein.

The effect of diclofenac on apo-hCox-2 fluorescence is both concentration and time dependent. Upon the addition of 10 μM drug, an immediate (within the mixing time) decrease of fluorescence occurred, followed by a slow further decrease over a period of 30 s to a final stable value (Figure 3). When 1.0 μM diclofenac was added, the initial rapid decrease in fluorescence was absent, but the fluorescence declined slowly over 3 min to the same final value as in the presence of 10 μM diclofenac. The further addition of 10 μM diclofenac, 10 min following the initial addition, in which time a stable fluorescence reading had been reached, had no further effect on the protein fluorescence intensity (data not shown). Time course measurements of tryptophan quenching were performed measuring total fluorescence in order to minimize protein consumption. This is justified since the contribution from tyrosine is minor.

The slow decrease in fluorescence intensity caused by diclofenac followed an exponential decay, from which the observed first-order rate constant for the process (k_{obs}) was calculated. Titrations were performed in which a range of diclofenac concentrations (0.5−20 μM) was added to apo-hCox-2. Both the magnitude of the initial rapid fluorescence loss ($F_0 - F$) and the first-order rate constant for the slow fluorescence decrease (k_{obs}) increased in a saturation-type manner (Figure 4).

The inhibition of Cox activity by time dependent inhibitors, such as diclofenac, has been shown to occur via a two-step process (Kulmacz & Lands, 1985; Copeland et al., 1994; Ouellet & Percival, 1995) in which the rapid formation of an initial reversible complex formation (EI) is followed by a slow isomerization to an essentially irreversibly bound form (EI*) (Scheme 1).

It would appear that the initial rapid and further slow decrease in intrinsic apo-hCox-2 fluorescence induced by diclofenac correspond with the two-step inhibition of Cox.

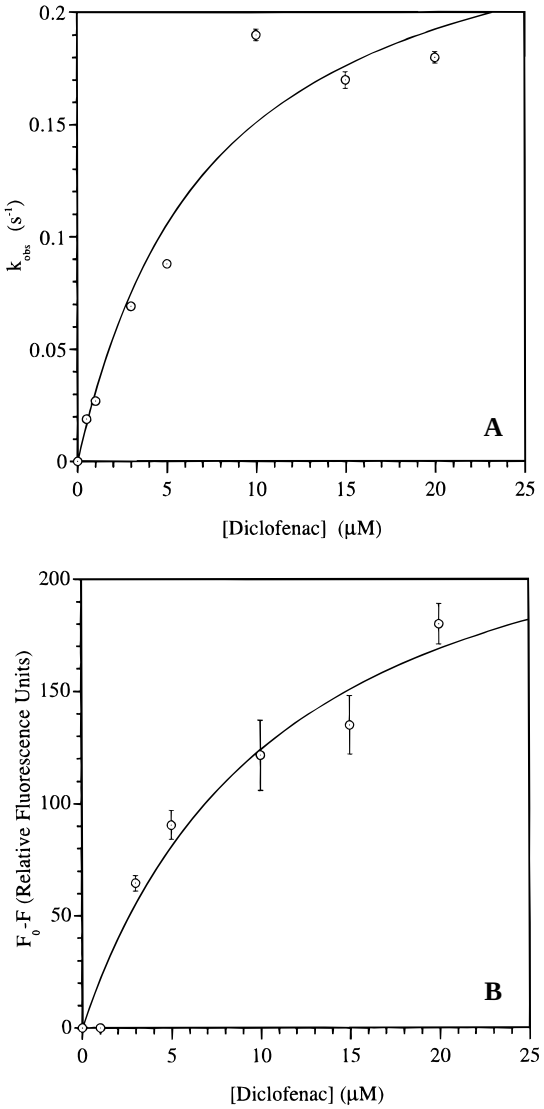

FIGURE 4: Effect of diclofenac concentration on apo-hCox-2 fluorescence changes. Apo-hCox-2 (18 μg/mL) was treated with 0−20 μM diclofenac, and the protein fluorescence was followed with time. The excitation and emission wavelengths were 280 and 325 nm, respectively. (A) Effect of diclofenac concentration on the observed first-order rate constant (k_{obs}) for the slow quenching of protein fluorescence. The slow decay of protein fluorescence at each diclofenac concentration was fitted to a first-order equation to obtain k_{obs} (see Materials and Methods). The error bars represent the standard error of the fit. The k_{obs} values were then computer fitted to the equation $k_{obs} = k_{on}[I]/(K_i + [I])$ to give calculated values of K_i and k_{on} of 7.6 ± 3.1 μM and 0.27 ± 0.05 s^{-1}, respectively. (B) Effect of diclofenac concentration on the rapid quenching of protein fluorescence. The magnitude of the rapid fluorescence decrease ($F_0 - F$) occurring within the mixing time (1 s) at each diclofenac concentration was computer fitted to the equation $(F_0 - F) = \Delta F_{max}/(1 + (K_i/[I]))$ to give a calculated value of K_i of 13.7 ± 4.7 μM and ΔF_{max} of 265 ± 53 FU. The values are the average of two determinations, and the error bars represent the range.

Scheme 1

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \xrightarrow{k_{on}} EI^*$$

As noted above, the magnitude of both phases of the inhibitor-induced fluorescence quenching increased in a saturation-type manner. Thus, the initial rapid fluorescence decrease likely reflects the formation of the rapidly reversible complex EI, while the slow time dependent fluorescence decrease reflects the accumulation of the irreversible complex EI*.

The values of the derived first-order rate constants for the slow loss of fluorescence (k_{obs}) at each inhibitor concentration ([I]) were computer fitted to the equation predicted by the model in Scheme 1 for slow irreversible inhibition (Kulmacz & Lands, 1985; Morrison & Walsh, 1987; see Materials and Methods) giving values of K_i and k_{on} of 7.6 ± 3.1 μM and 0.27 ± 0.05 s^{-1}, respectively (Figure 4A). The value of K_i was also estimated by fitting the data for the magnitude of the rapid fluorescence decrease ($F_0 - F$) versus inhibitor concentration (Figure 4B). This value ($K_i = 11.3 \pm 4.7$ μM) is in good agreement with that obtained above. Comparable values of K_i and k_{on} for diclofenac binding (24 ± 12 μM and 0.19 ± 0.05 s^{-1}, respectively) were also obtained by determination of the observed rate constants for the onset of inhibition of enzyme activity (see Materials and Methods), thereby clearly linking the observed intrinsic protein fluorescence changes with inhibitor binding. From the data in Figure 4B, the magnitude of the rapid fluorescence decrease ($F_0 - F$) at saturating diclofenac concentration can be calculated as 264 ± 53 FU. This value is close to the total fluorescence quenching observed in the presence of diclofenac (Figure 3, 240 FU). Since it was established above that the rapid fluorescence decrease reflects the formation of EI and the slow time dependent fluorescence decrease reflects the accumulation of EI*, these results indicate that both the EI and the EI* complexes have the same intrinsic fluorescence, which is approximately 50% that of the native enzyme.

The possibility can be considered that the above inhibitor-induced apo-hCox-2 fluorescence changes are due to the presence of multiple sites, binding at one inducing the rapid fluorescence change and at a second causing the slow fluorescence decrease. The stoichiometry of apo-hCox-2−diclofenac binding was determined by titrating the cyclooxygenase activity of a fixed concentration of enzyme (3.4 μM) with increasing diclofenac concentrations (0−4 μM). The activity was determined after 15 min of preincubation, after which time no further increase in inhibition was observed. The cyclooxygenase activity of apo-hCox-2 decreased linearly with increasing inhibitor concentration ($R = 0.98$), giving a value of 3.8 μM diclofenac for 100% inhibition. This value is close to the stoichiometry of 1:1 expected for the mechanism shown in Scheme 1. The effect of diclofenac concentration on the fluorescence quenching of apo-hCox-2 was also consistent with a 1:1 stoichiometry. Addition of diclofenac (0.24 μM) to apo-hCox-2 (18 μg/mL, 0.25 μM) resulted in the slow decrease in fluorescence ($\lambda_{em} = 280$ nm, $\lambda_{ex} = 325$ nm) to the same final value as obtained with higher inhibitor concentrations. Finally, only a single inhibitor binding site was found in the X-ray crystal structure of oCox-1 (Picot et al., 1994). The above results are therefore inconsistent with the presence of multiple high-affinity sites.

NSAIDs represent a wide diversity of chemical structures (Figure 5). Each of the time dependent NSAIDs, representing five structural classes, was tested for its ability to quench apo-hCox-2 tryptophan fluorescence. All of the time dependent inhibitors caused an effect similar to that observed with diclofenac, that is, a rapid fluorescence decrease followed by a slow further decline to a final stable value. The total magnitude of the fluorescence quenching and the spectral shift caused by each inhibitor is shown in Table 1.

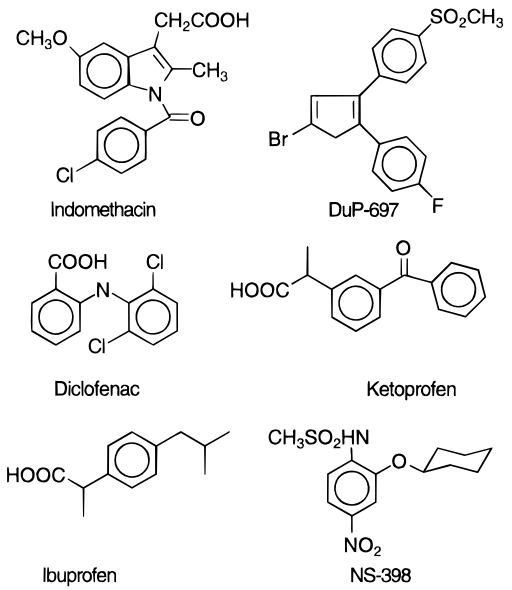

FIGURE 5: NSAIDs used in this study. Indomethacin, diclofenac, NS-398, and ketoprofen are time dependent, effectively irreversible inhibitors of hCox-2. Ibuprofen is a reversible inhibitor of hCox-2.

Table 2: Kinetic Constants for the Interaction of NSAIDs with hCox-2 Measured by Fluorescence Titration and Enzyme Inhibition

	fluorescence titration		enzyme inhibition	
inhibitor	K_i (μM)	k_{on} (s^{-1})	K_i (μM)	k_{on} (s^{-1})
diclofenac	7.6 ± 3.1	0.27 ± 0.05	24 ± 12	0.19 ± 0.05
NS-398	33.5 ± 9.5	0.38 ± 0.08	47 ± 30	0.22 ± 0.12
ketoprofen	16.9 ± 5.3	0.52 ± 0.10	20 ± 12	0.08 ± 0.03
DuP-697[a]	0.17 ± 0.02	0.033 ± 0.001	0.9 ± 0.2	0.14 ± 0.02

[a] The kinetic constants for DuP-697 were determined by fluorescence titration in the presence of 2 μM ibuprofen.

The values of K_i and k_{on} for the binding of each inhibitor to apo-hCox-2 were obtained by titrations of both the time dependency of the slow fluorescence quenching and the inhibition of hCox-2 activity as described above (Table 2). As in the case of diclofenac, there is fairly good agreement between the two methods. A fluorescence titration could not be performed with indomethacin above 10 μM due to its high absorption ($\epsilon_{280nm} = 15\,200\ M^{-1}\ cm^{-1}$). Up to this concentration, no saturation-type effect on the observed first-order rate constants for the slow decrease in protein fluorescence (k_{obs}) could be detected. This is consistent with the previously determined K_i value of 114 μM for indomethacin (Ouellet & Percival, 1995). DuP-697 could not be titrated under the present conditions as initial investigations showed the enzyme to be close to saturation at 0.5 μM inhibitor concentration. Lower concentrations would lead to inhibitor depletion as the enzyme concentration was 0.24 μM. Two other commonly used time dependent inhibitors from the aryl acetic acid series, flurbiprofen and naproxen, demonstrate fluorescence coincident with that of hCox-2, thereby interfering with analysis of their behavior. The value of k_{on} obtained for NS-398 (Table 2) is on the order of 10-fold higher than that measured previously from enzyme inhibition data (Ouellet & Percival, 1995). We have no explanation for this discrepancy.

The above measurements of inhibitor binding constants by fluorescence were performed using apo-hCox-2, whereas those determined by enzyme inhibition were obtained with holoenzyme. It is therefore of importance to determine whether the presence of heme has an effect on the kinetics of inhibitor binding. Fluorescence titrations of holo-hCox-2 were not possible since the Trp fluorescence is quenched 65% due to energy transfer to the heme (Stryer, 1978). The addition of 1.0 μM diclofenac resulted in less than a 10% decrease in holo-hCox-2 fluorescence (results not shown). Inhibitor binding to apo-hCox-2 and holo-hCox-2 was determined from the rate of onset of inhibition at a single inhibitor concentration as described in Materials and Methods. Apo- and holoenzyme were preincubated with inhibitor for 5−600 s, prior to the initiation of the reaction with substrate, and for apoenzyme heme. The observed rate constants for the onset of inhibition for apo- and holo-hCox-2, respectively, were as follows: diclofenac (1.0 μM), $0.005 \pm 0.0015\ s^{-1}$, $0.0038 \pm 0.0005\ s^{-1}$; NS398 (2.0 μM), $0.010 \pm 0.002\ s^{-1}$, $0.006 \pm 0.001\ s^{-1}$; ketoprofen (2.0 μM), $0.009 \pm 0.002\ s^{-1}$, $0.0035 \pm 0.0005\ s^{-1}$; DuP-697 (0.1 μM), $0.015 \pm 0.004\ s^{-1}$, $0.011 \pm 0.002\ s^{-1}$. The values for each inhibitor are within or close to experimental error with the exception of ketoprofen, where inhibition of apoenzyme is approximately 2.5-fold faster than holoenzyme. Thus, the presence of heme does not significantly affect the binding of these four inhibitors to hCox-2. This is in apparent contrast to the inhibition of oCox-1 with ASA, in which case the holoenzyme is acetylated approximately 100-fold faster than apoenzyme (Kulmacz, 1989). The difference could be the result of the mechanism of inhibition, namely, time dependent non-covalent versus covalent.

Some indication of nonspecific binding of DuP-697 and NS-398 to apo-hCox-2 was detected. The addition of drug to enzyme previously treated with an excess of inhibitor resulted in an instantaneous further decrease in protein fluorescence. The magnitude of this effect was small, approximately 100 FU with 10 μM DuP-697, compared to an initial decrease of 356 FU in the presence of 0.5 μM DuP-697. The addition of lower concentrations of DuP-697 (after the initial 0.5 μM DuP-697) resulted in smaller decreases in fluorescence. This effect is likely due to weaker nonspecific binding to other hydrophobic regions of the protein, since the activity titration of diclofenac stoichiometry described above was consistent with a single inhibitor binding site.

Effect of Ibuprofen and Docosahexaenoic Acid on Apo-hCox-2 Fluorescence. The NSAID ibuprofen (Figure 5) and the fatty acid docosahexaenoic acid (22:6, $n - 3$) showed no significant effect on the intrinsic fluorescence of apo-hCox-2 at a concentration of 3 μM (Figure 6, line A). Since they are both competitive, reversible inhibitors (Rome & Lands, 1975; Kulmacz & Lands, 1985; Smith et al., 1995), their effects on the fluorescence quenching of apo-hCox-2 by the time dependent inhibitor diclofenac was investigated. Treatment of apo-hCox-2 with 0.5 μM ibuprofen prior to the addition of 10 μM diclofenac (Figure 6, line C), compared to 10 μM diclofenac alone (Figure 6, line D), resulted in a reduction in both the magnitude of the initial rapid fluorescence decrease (120 FU compared to 230 FU) and the rate constant for the slow fluorescence decline ($0.024\ s^{-1}$ compared to $0.11\ s^{-1}$). The inclusion of 3 μM ibuprofen resulted in the near abolition of the rapid fluorescence decrease and a further reduction in k_{obs} to $0.015\ s^{-1}$ (Figure 6, line B). Qualitatively similar effects were obtained with docosahexaenoic acid on the diclofenac-induced quenching of apo-hCox-2 fluorescence (results not shown). These

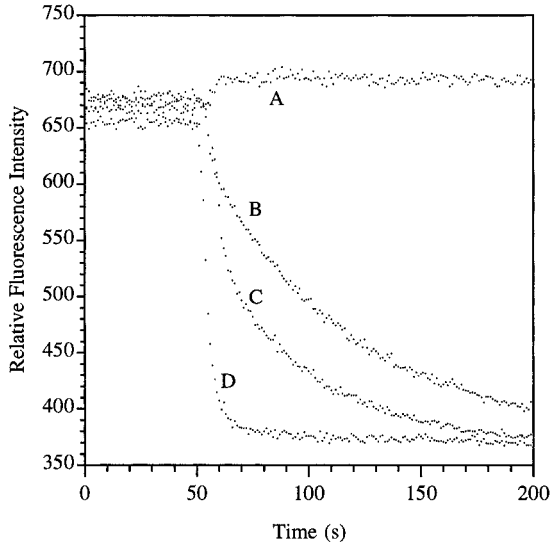

FIGURE 6: Effect of ibuprofen on the diclofenac-induced fluorescence quenching of apo-hCox-2. Apo-hCox-2 (18 μg/mL) was pretreated with different concentrations of ibuprofen followed by the addition (at time 50 s) of 10 μM diclofenac. (A) 3 μM ibuprofen alone, added at 50 s (no diclofenac); (B) 3 μM ibuprofen; (C) 0.5 μM ibuprofen; (D) 0 μM ibuprofen. Data points after the addition of diclofenac were multiplied by 1.05 to account for the inner filter effect. The excitation and emission wavelengths were 280 and 325 nm, respectively. The mixing time was 5 s, and the data interval was 1 s.

results are therefore consistent with the competitive behavior of both ibuprofen and docosahexaenoic acid toward diclofenac. The results are also consistent with the previous conclusion that both the initial enzyme−diclofenac (EI) complex and the irreversibly inhibited complex (EI*) have a reduced intrinsic fluorescence. The effect of a reversible, competing inhibitor (such as ibuprofen or docosahexaenoic acid) in the presence of the irreversible inhibitor diclofenac (I) acting via a mechanism as in Scheme 1 would be to reduce the fraction of enzyme initially in the EI form and therefore also reduce the rate of accumulation of EI*.

Estimates of the K_i values of ibuprofen and docosahexaenoic acid for apo-hCox-2 were obtained from their effects on the rate constants for the slow time dependent fluorescence decrease due to diclofenac (see Materials and Methods). These values, 0.2 ± 0.1 and 0.2 ± 0.09 μM for ibuprofen and docosahexaenoic acid, respectively, are similar to those obtained from enzyme inhibition studies of ibuprofen with hCox-2 (0.92 ± 0.35 μM; Rider et al., 1996) and docosahexaenoic acid with oCox-1 (0.19−0.67 μM; Kulmacz & Lands, 1985).

Ibuprofen also showed the same competitive behavior (data not shown) toward the other time dependent NSAIDs in Figure 5. K_i values of 0.38 and 0.22 μM were determined for ibuprofen in the presence of NS-398 and ketoprofen, respectively. The competitive behavior of ibuprofen allowed an estimate to be made of the kinetic constants of DuP-697 binding to apo-hCox-2. Apo-hCox-2 was titrated with 0.5−6.0 μM DuP-697 in the presence of 2 μM ibuprofen, and k_{obs} values for the slow fluorescence decrease was determined. The presence of the competing ibuprofen raised the apparent K_i for DuP-679 so that saturation was achieved at a higher inhibitor concentration, thereby avoiding the previous problem of inhibitor depletion. Computer fitting of the data (see Materials and Methods) gave values of K_i and k_{on} of 0.17 ± 0.01 μM and 0.033 ± 0.001 s^{-1}, respectively, which are in fair agreement with those from inhibition studies (Table 2).

Table 3: Relative Fluorescence Intensities (% *F*) of Native and Diclofenac-Inhibited apo-hCox-2 in the Presence of Collisional Quenchers

	fluorescence intensity (%)[a]	
quenching agent	native apo-hCox-2	diclofenac-inhibited apo-hCox-2
none	100	53
0.3 M CsCl	100	53
0.3 M KI	94	47
0.3 M acrylamide	86	45

[a] Fluoresence intensities were measured at excitation and emission wavelengths of 280 and 327 nm, respectively.

Effect of Collisional Quenchers on Apo-hCox-2 Fluorescence. To determine any change in solvent accessibility of tryptophan residues on inhibitor binding, the effects of the ionic collisional quenchers KI and CsCl and the non-ionic hydrophilic quencher acrylamide were tested. To account for any ionic strength effects KCl was used as a control. In the cases of both KCl and CsCl, concentrations of up to 0.3 M had no detectable effect on the total protein and tryptophan fluorescence emission maxima or intensities of either the native apo-hCox-2 or the diclofenac-inhibited enzyme. For KI, at 0.3 M, the total fluorescence intensity of the native apoenzyme was quenched by 6% (Table 3). Diclofenac-inhibited enzyme exhibited a fluorescence quenching in the presence of 0.3 M KI of the same absolute magnitude as that of the native apoenzyme. This quenching represents 12% of the intrinsic fluorescence, since the fluorescence intensity of the inhibited enzyme is approximately 50% that of the native enzyme (Table 3). No shift in the emission maximum was detected. These results suggest that the same tryptophan residues are quenched in both the native and inhibited enzyme and that these residues are not affected by diclofenac binding and are still accessible to solvent to the same degree. Higher concentrations of KI were not used because of possible chaotropic effects (Ide & Engelborghs, 1981). Similar results were obtained with 0.3 M acrylamide (Table 3), although both native and inhibited forms were quenched equally (18% and 15%, respectively). As noted above, the inhibited form is quenched approximately 50% compared to the native enzyme, hence the magnitude of the acrylamide-induced quenching is less than that for the native enzyme. This indicates that the tryptophan residues quenched in the native enzyme become somewhat less accessible to acrylamide upon diclofenac binding.

The effects of the collisional quenching agents employed here are small and indicate that the apo-hCox-2 tryptophan residues that contribute to the majority of the protein fluorescence are buried in the protein and are not solvent accessible, in either native or inhibited forms, to an appreciable degree.

Modification of Tryptophan Residues. Protein modification studies using DHNBS were conducted to further explore the solvent exposure of apo-hCox-2 tryptophan residues. Treatment of apo-hCox-2 and indomethacin-inhibited apo-hCox-2 with a 100-fold excess of DHNBS for 10 min, conditions which lead to modification of apparently solvent accessible tryptophan residues in other proteins (Werber et al., 1987; Horton & Tucker, 1970), did not result in any

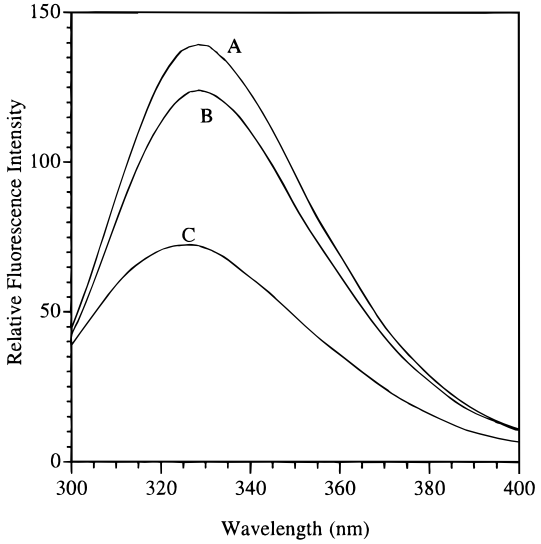

FIGURE 7: Effect of NS-398 on the fluorescence emission spectrum of ASA-acetylated apo-hCox-2. (A) Acetylated apo-hCox-2; (B) apo-hCox-2; (C) acetylated apo-hCox-2 treated with 1.0 μM NS-398 for 10 min. The protein concentration was 18 μg/mL, and the excitation wavelength was 280 nm.

detectable protein modification. The lack of tryptophan accessibility in the native protein was confirmed in experiments in which a 1000-fold excess of reagent also gave no modification. However, approximately five protein residues were modified using a 1000-fold excess of DHNBS and SDS-denatured apo-hCox-2.

Effect of ASA-Acetylation on Apo-hCox-2 Fluorescence. Acetylated apo-hCox-2 has a slightly increased (18%) total fluorescence compared to that of the native apoenzyme (Figure 7). The tryptophan emission spectrum maximum is also unshifted from the native enzyme and is increased in intensity to the same degree. Upon addition of 1 μM NS-398, the protein fluorescence was quenched in the same time dependent manner as for the native enzyme and the k_{obs} for this process was within 30% of the value obtained for the non-acetylated enzyme (Figure 8A). The magnitude of the fluorescence quenching, as a percentage of the initial value, was the same as that of the native enzyme (Figure 7). No significant differences in k_{obs} values (within 10%) for the slow fluorescence quenching were detected in the effects of the time dependent inhibitors indomethacin, ketoprofen, and DuP-697 (at 1 μM) on the two forms of the enzyme. In contrast, diclofenac caused only a small instantaneous reduction (3%) in the fluorescence of the acetylated enzyme but, as detailed above, quenched the native apoenzyme in a time dependent fashion by approximately 50% (Figure 8B). Clearly, these results point to a difference in the binding of diclofenac to acetylated and native hCox-2, whereas no difference is evident for the other time dependent NSAIDs tested.

Ibuprofen had no effect on the intrinsic fluorescence of both the native and acetylated enzyme. A measure of the interaction of ibuprofen with the acetylated enzyme was obtained from its effect on the rate constant for the slow fluorescence decrease due to ketoprofen. The k_{obs} value decreased from 0.011 $\pm$ 0.007 s^{-1} in the presence of 3 μM ibuprofen and 5 μM ketoprofen for native enzyme to 0.0037 $\pm$ 0.0007 s^{-1} for acetylated enzyme. The K_i value of 0.066 μM for ibuprofen was calculated (see Materials and Methods), showing that ibuprofen binds 3-fold more tightly to the acetylated protein than the native form.

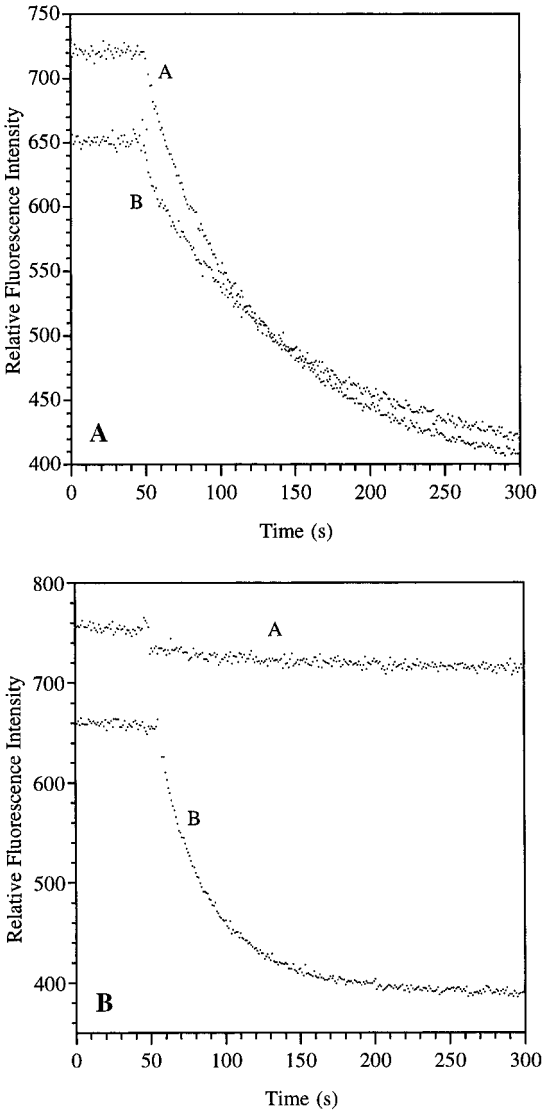

FIGURE 8: Effect of NS-398 and diclofenac on acetylated apo-hCox-2 fluorescence. Acetylated apo-hCox-2 and native apo-hCox-2 (both 18 μg/mL) were treated at time 50 s with 1 μM inhibitor. (A) NS-398, line A, acetylated apo-hCox-2; line B, apo-hCox-2. (B) Diclofenac, line A, acetylated apo-hCox-2; line B, apo-hCox-2. The excitation and emission wavelengths were 280 and 325 nm, respectively. The mixing time was 5 s, and the data interval was 1 s.

Estimation of Fluorescence Quenching Due to Energy Transfer to Inhibitors. hCox-2 contains six tryptophan residues, and hence the intrinsic protein fluorescence is expected to reflect the average of the contributions from each of these residues in their different environments. Any change in the environment of individual tryptophan residues could result in an alteration of fluorescence characteristics such as emission wavelength, quantum yield, and susceptibility to quenching (Pawagi & Deber, 1990). Fluorescence quenching can also result from energy transfer to an acceptor molecule having an overlapping absorption spectrum (Cheung, 1991). Each of the inhibitors that caused fluorescence quenching has an absorption spectrum that overlaps, at least partially, with the fluorescence emission spectrum of apo-hCox-2. Both ibuprofen and docosahexaenoic acid, which did not cause fluorescence quenching, have no absorption in the region of 290−400 nm. To determine whether energy transfer could

Table 4: Spectral Overlap Integrals and R_0 Values for apo-hCox-2 Fluorescence Energy Transfer to Inhibitors

inhibitor	$J \times 10^{13}$ (M^{-1} cm^{-1} nm^4)	R_0 (Å)
indomethacin	6.06	24
NS-398	5.90	24
DuP-697	9.90	25
ketoprofen	0.48	15
diclofenac	0.50	16

Table 5: Relative Quantum Yields (Q_p/Q_a) of Individual Trp Residues and Apo-hCox-2 in the Presence of Inhibitors with R_0 Values of 15 and 24 Å

	r (Å)[a]	Q_p/Q_a ($R_0 = 24$ Å)	Q_p/Q_a ($R_0 = 15$ Å)
Trp86[b]	12	0.02	0.21
Trp125	30	0.79	0.98
Trp291	14	0.04	0.40
Trp309	31	0.82	0.99
Trp373	<5	0.00	0.00
Trp531	19	0.20	0.80
protein[c]		0.31	0.56

[a] Distance between (*S*)-flurbiprofen binding site and each tryptophan indole ring in oCox-1. [b] hCox-2 residue numbers. The corresponding oCox-1 residue numbers are obtained by adding 14. [c] The relative quantum yield of inhibited apo-hCox-2 was calculated from the equation $(Q_p/Q_a)_{protein} = \Sigma(Q_p/Q_a)/6$.

account for the observed inhibitor-induced quenching, the relative quantum yield of apo-hCox-2 in the presence of each inhibitor was calculated (Cheung, 1991; Fox et al., 1993) using inhibitor−tryptophan distances obtained from the crystal structure model of oCox-1 (Picot et al., 1994; Garavito et al., 1995). Each of the six tryptophan residues in hCox-2 is conserved in oCox-1, and because of their 61% amino acid identity, it is reasonable to assume that the two enzymes have similar structures and that the inhibitor binding site is similarly located in both enzymes. Calculation of the spectral overlap integral, *J*, for each inhibitor (Table 4) shows that the donor−acceptor distances resulting in 50% donor quenching, R_0, are either approximately 15 or 24 Å for all five inhibitors. Using these two R_0 values and the distances from each tryptophan to the inhibitor binding site (Picot et al., 1994; Garavito et al., 1995), the relative quantum yield, Q_p/Q_a, of each tryptophan and the protein, in the presence of inhibitor, can be calculated (Fox et al., 1993). The results (Table 5) show that even for the inhibitors having the weakest spectral overlap, quenching on the order of 44% may be expected, indicating that energy transfer could account for the observed effects.

DISCUSSION

Time dependent inhibitors of Cox operate by a two-step mechanism shown in Scheme 1 (Kulmacz & Lands, 1985). Each of the time dependent inhibitors tested in this study caused a fluorescence quenching of apo-hCox-2 that was clearly separable into two processes. Each inhibitor induced a rapid fluorescence quenching (within the mixing time) that was followed by a slower decline (over 0.5−10 min) to a constant final value (Figure 3). The magnitude of the initial rapid fluorescence quenching and the rate constant for the slow decline both increased with inhibitor concentration in a saturation-type manner (Figure 4). Therefore, the rapid fluorescence loss results from the formation of the reversible EI complex, and the further slower decrease follows the accumulation of the tightly bound EI* complex (Scheme 1). There appears to be little, if any, difference in fluorescence between the two complexes (EI and EI*). The kinetic constants derived for the inhibitor binding process, K_i and k_{on}, from the fluorescence changes are in good agreement with those obtained from enzyme activity measurements, thereby clearly linking the ligand-induced fluorescence changes to the onset of enzyme inhibition (Table 2).

The reversible, time independent inhibitors ibuprofen and docosahexaenoic acid did not cause protein fluorescence quenching, but their effects on fluorescence changes induced by the time dependent inhibitor diclofenac (Figure 6) is consistent with their binding and competitive behavior. Ibuprofen is competitive toward all five of the time dependent inhibitors tested, suggesting that, although these compounds are structurally distinct, their individual binding sites are common or overlap to some degree.

The results with the collisional quenching agents I^-, Cs^+, and acrylamide indicate that the tryptophan residues responsible for the majority of apo-hCox-2 fluorescence are buried in the protein matrix and are not solvent accessible in either the native or inhibited forms. The blue-shifted tryptophan emission of the native protein and the lack of modification by the tryptophan reagent DHNBS are also consistent with the low degree of solvent accessibility. The lack of any major change in the susceptibility of the inhibited enzyme to quenching agents is therefore inconsistent with an inhibitor-induced protein conformational change resulting in increased solvent accessibility of the tryptophan residues.

ASA-acetylated apo-hCox-2 has a similar fluorescence emission spectrum to the native protein. All of the time dependent inhibitors tested, with the exception of diclofenac, caused a time dependent fluorescence quenching of the acetylated enzyme with the same overall magnitude and similar k_{obs} values as for the native protein. This is evidence that the acetylation does not result in a blocking of the NSAID binding site or a protein conformational change leading to the loss of the inhibitor binding site. For diclofenac there was no evidence of inhibitor binding to the acetylated apo-hCox-2 (Figure 8B). This result is of interest as ibuprofen was shown to bind apo-hCox-2 competitively with all five time dependent NSAIDs used in this study, including diclofenac. As noted above, this suggests that their binding sites overlap to some degree. The fact that binding of NSAIDs other than diclofenac is not affected by acetylation strongly suggests that the diclofenac binding site is somehow distinct and is blocked by ASA-acetylation. The site of ASA-acetylation in hCox-2 has been determined as Ser516 (Wennogle et al., 1995), which corresponds to Ser530 in oCox-1. The X-ray crystal structure of bromoacetylated oCox-1 (Loll et al., 1995) shows that the major bromoacetyl rotameric conformer occupies a space partially overlapping that filled by flurbiprofen and blocking the channel to the cyclooxygenase active site. A minor bromoacetyl conformer is folded back into an alcove and does not completely block this channel. Since the amino acids lining the cyclooxygenase active site are highly conserved between Cox-1 and Cox-2 (Loll et al., 1995), it is likely that a similar situation occurs with Cox-2. Therefore NSAID binding to acetylated hCox-2 probably occurs with the acetyl group in the minor folded back conformation. It can be further speculated that the diclofenac binding site overlaps to some extent with the above-mentioned alcove, which is filled by the acetyl group,

since the acetyl and diclofenac cannot be accommodated at the same time.

ASA-acetylated Cox-2 catalyzes the formation of 15-HETE, in contrast to acetylated Cox-1, which is catalytically inactive (Holtzman et al., 1992; Lecomte et al., 1994). The binding of NSAIDs to acetylated hCox-2 can therefore be measured from the inhibition of 15-HETE synthase activity. Such titrations show that the IC_{50} value of diclofenac is increased greater than 500-fold for ASA-acetylated hCox-2 over native enzyme, whereas NSAIDs from other structural classes were largely unaffected (J. Mancini, unpublished results).

It is difficult to determine unequivocally the physical cause of the inhibitor-induced fluorescence quenching of apo-hCox-2. It is likely due to energy transfer to the bound inhibitor, as calculations based on tryptophan-inhibitor distances in oCox-1 indicate that at least an approximately 44% decrease in quantum yield is possible with the inhibitors tested (Table 5). In addition, the two inhibitors that do not induce a quenching have no absorption in the region of the protein fluorescence emission and hence energy transfer cannot occur.

A second possibility involves a protein conformational change occurring on inhibitor binding resulting in a 50% reduction in the fluorescence quantum yield. Evidence of a protein conformational change induced by certain NSAIDs and heme is available from proteolysis studies of oCox-1. The oCox-1 apoenzyme is cleaved by high concentrations of trypsin into two polypeptides, but the holoenzyme and inhibitor-treated enzyme are resistant to proteolysis (Chen et al., 1987; Kulmacz, 1989). The crystal structure of oCox-1 shows that the binding site of the NSAID (*S*)-flurbiprofen is within a hydrophobic channel leading to the cyclooxygenase active site (Picot et al., 1994), whereas the loop containing the trypsin cleavage site at Arg253 is on the exterior of the protein and on the opposite side from the hydrophobic channel. Therefore, the NSAID-induced protection is likely due to a protein conformational change rather than a simple blocking of protease access. The trypsin cleavage site at Arg253 is not present in hCox-2, and detergent solubilized apo-hCox-2 is highly resistant to trypsin and other proteases (V. Houtzager, unpublished data). Consequently, protease sensitivity cannot be used to monitor any conformational changes induced in hCox-2 by inhibitor binding. Ibuprofen does not cause any fluorescence quenching of apo-hCox-2 (Figure 6) but has been shown to protect against trypsin cleavage and therefore cause a protein conformational change (Kulmacz, 1989). This would argue against the inhibitor-induced fluorescence quenching of apo-hCox-2 being due to a protein conformational change.

From the oCox-1 crystal structure (Picot et al., 1994), and assuming a similar structure for hCox-2, a single tryptophan residue (373) is located in the active site of hCox-2, close to Tyr371, the residue likely responsible for the H atom abstraction from arachidonic acid (Hsi et al., 1994). A second tryptophan (291) is also buried in a hydrophobic environment in the catalytic domain, whereas the others appear to be either partially or completely solvent exposed. The possibility exists that the two buried tryptophans contribute to the majority of the intrinsic protein fluorescence in the native protein, while the four exposed residues are quenched by solvent or other mechanism. The structures of the (*S*)-flurbiprofen− (Picot et al., 1994) and indomethacin−oCox-1 (Garavito et al., 1995) complexes show the inhibitor positioned close to Trp373 which could result in its quenching due to an environmental perturbation. This would explain the 40%−50% fluorescence decrease observed on inhibitor binding. However, this hypothesis could not be confirmed, as the apparently solvent-exposed or partially exposed residues were not modified using the tryptophan specific reagent DHNBS. The lack of hCox-2 tryptophan modification by DHNBS is likely linked to a masking of these residues from the reagent. Closer analysis of the oCox-1 crystal structure (Picot et al., 1994) shows that three exposed residues are on the interface of the monomer−monomer interaction and that their exposed surface is greatly reduced by the presence of the other monomer. The present results are therefore consistent with hCox-2 existing as a dimer in the detergent-solubilized form. The fourth exposed Trp is situated in the amphipathic helices which are believed to anchor the enzyme to the lipid bilayer (Picot et al., 1994). It would not be unexpected that this region is masked by bound detergent and hence inaccessible to DHNBS.

Whatever the physical explanation for the quenching process, it is apparent that the inhibitor-induced fluorescence quenching follows the formation of both the reversible enzyme−inhibitor complex (EI) and the tightly bound complex (EI*), but not the tightly bound form (EI*) alone. Therefore, the kinetically observable isomerization of EI to EI* does not involve a major change in the inhibitor binding mode as this would likely result in a change in tryptophan fluorescence. The results would therefore argue against a mechanism in which the inhibitor binds to an initial site, perhaps lower in the hydrophobic channel leading to the active site, and then slowly moves up past the constriction at Arg120-Tyr355 (oCox-1 residue numbers; Picot et al., 1994) to the final binding site near Tyr385.

The results of this study demonstrate that NSAID binding to the apoenzyme form of hCox-2 can be conveniently followed by fluorescence spectroscopy. This technique should be useful for the evaluation of inhibitor kinetic constants in the absence of enzyme turnover and also for the further characterization of the mechanism of cyclooxygenase inhibition by both reversible and irreversible inhibitors.

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