Kinetics of the Interaction of Nonsteroidal Antiinflammatory Drugs with Prostaglandin Endoperoxide Synthase-1 Studied by Limited Proteolysis†

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ABSTRACT: Many nonsteroidal antiinflammatory agents (NSAIDs) bind to prostaglandin endoperoxide synthase (PGHS) and induce a conformational change in the PGHS apoprotein that renders it resistant to cleavage by trypsin at Arg^{277}. In the present study, the trypsin protection assay was modified to permit detection of conformational changes at times as short as 5 s after the addition of inhibitor. The kinetics of the induction and reversal of trypsin resistance in apoPGHS-1 by a series of NSAIDs and isozyme-specific PGHS-1 and PGHS-2 inhibitors were determined. All compounds induced resistance to trypsin cleavage at a rapid rate. The conformational change induced by competitive inhibitors was reversed on prolonged incubation with trypsin (∼5 min). In contrast, the resistance induced by irreversible inhibitors was not lost during a 5 min incubation with trypsin. All of the selective PGHS-2 inhibitors protected against tryptic cleavage of apoPGHS-1 but did not inhibit the protein's cyclooxygenase activity. The results suggest that induction of trypsin resistance is a reflection of the initial association of reversible as well as irreversible inhibitors with the apoprotein.

A major determinant of the pharmacological activity of nonsteroidal antiinflammatory drugs (NSAIDs)[1] is their ability to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase (PGHS) (Vane, 1971). Inhibition of the cyclooxygenase prevents synthesis of PGE_2 which is an important mediator of inflammation and hyperalgesia (Ferreira et al., 1978). The discovery of a second gene for PGHS (PGHS-2) that is expressed as an immediate-early gene in inflammatory cells and in the central nervous system has renewed interest in the interaction of NSAIDs with PGHS (Kujubu et al., 1991; Breder et al., 1995). Like the constitutively expressed PGHS-1, PGHS-2 exhibits both cyclooxygenase and peroxidase activities and is inhibited by all commercially available NSAIDs (Meade et al., 1993). The differential tissue distribution and the response to inducers displayed by the two forms of PGHS suggest that they play different physiological roles, which is substantiated by the separate phenotypes exhibited by transgenic animals bearing homozygous deletions of either PGHS-1 or PGHS-2 genes (Morham et al., 1995; Langenbach et al., 1995; Dinchuk et al., 1995). It has been proposed that PGHS-2 is responsible for inflammation and hyperalgesia, whereas PGHS-1 is responsible for gastrointestinal cytoprotection and platelet function (Masferrer et al., 1994). Thus, considerable effort has been expended toward the development of selective PGHS-2 inhibitors as candidate NSAIDs that lack the gastric irritant activity which is the major limiting side effect of all presently available NSAIDs (O'Neill et al., 1995).

The interaction of most NSAIDs with PGHSs follows a two-step sequence (eq 1) (Rome & Lands, 1975). The first

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E-I] \xrightarrow{k_2} [E-I]^* \qquad (1)$$

$$K_i = k_{-1}/k_1 \quad k_{inact} = (k_{-1} + k_2)/k_1$$

step is rapid and reversible and results in competitive inhibition of the enzyme. The second step is conversion of the initial enzyme−inhibitor complex to one in which the inhibitor is bound more tightly and functionally irreversibly. Some NSAIDs such as ibuprofen only inhibit PGHS competitively, whereas others such as indomethacin and flurbiprofen are slow, tight-binding inhibitors. Recent investigations with PGHS-2 selective inhibitors reveal that the slow conversion of the reversible complex to the functionally irreversible complex is responsible for the selectivity of inhibition of PGHS-2 over PGHS-1 and vice versa (Copeland et al., 1994).

The three-dimensional structure of PGHS-1 complexed with a molecule of the inhibitor flurbiprofen has been solved at 3.5 Å resolution (Picot et al., 1994). Membrane-binding domains, heme-binding domains, and substrate access channels for both the cyclooxygenase and peroxidase activities are evident in different locations on the protein. The cyclooxygenase and peroxidase active sites are positioned on opposite sides of the heme prosthetic group, and flurbiprofen is bound in the cyclooxygenase substrate channel. This explains why all NSAIDs inhibit cyclooxygenase but not peroxidase activity (Robinson & Vane, 1974).

PGHS-1 contains an exposed peptide loop from residue His^{274} to Gln^{284} that exhibits differential sensitivity to

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[1] Abbreviations: NSAID, nonsteroidal antiinflammatory drug; PGHS, prostaglandin endoperoxide synthase (EC 1.14.99.1); SQ 29,535, ([1*R*-[1α,2β(5*Z*), 3β,4α]]-7-[(hexyloxy)methyl]-7-oxabicyclo[2.2.1]hept-2-yl-2,2-dimethyl-5-heptenoic acid; SC 8076, 2-(2-chlorophenyl)-4-[4-(methylthio)phenyl]-5-[4-(methyloxy)phenyl]thiazole; SC 8092, 2-(2-chlorophenyl)-4-[4-(methylsulfonyl)phenyl]-5-[4-(methyloxy)-phenyl]thiazole; TAME, *N*-α-*p*-tosyl-L-arginine methyl ester; TPCK, L-1-tosylamide 2-phenylethyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethyl sulfoxide.

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proteolytic cleavage when the heme prosthetic group or inhibitors are bound (Kulmacz, 1989). For example, the peptide bond at Arg^{277} is cleaved by trypsin in the apoprotein but not in the holoprotein and not in the apoprotein bound with NSAIDs (Kulmacz & Lands, 1982; Chen et al., 1987; Kulmacz, 1989). Thus, the extent of trypsin resistance of PGHS-1 is a sensitive indicator of protein−inhibitor binding. The kinetics of induction of trypsin resistance have not been determined so it is not known whether the conformational change that it reflects is associated with the formation of the initial inhibitor−PGHS-1 complex depicted in eq 1 or the tightly bound inhibitor−PGHS-1 complex formed from it. Therefore, we have modified the trypsin cleavage assay to permit the detection of conformational changes as early as 5 s after the addition of inhibitor. Using this assay, we have determined the kinetics of induction of trypsin resistance by several classic NSAIDs and some novel PGHS-1 and PGHS-2 selective inhibitors. In addition, we have further modifed the assay to detect reversibility of inhibitor binding. The results of the experiments indicate that the trypsin protection assay can be successfully employed to monitor inhibitor binding and release and that the induction of trypsin resistance by an inhibitor reflects its initial association with the enzyme.

MATERIALS AND METHODS

Arachidonic acid was purchased from Nu Chek Prep (Elysian, MN). [1-^{14}C]Arachidonic acid (57 mCi/mmol) was purchased from NEN Dupont. Hematin, bovine pancreas trypsin/L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), *N*-α-*p*-tosyl-L-arginine methyl ester (TAME), indomethacin, ibuprofen, and mefenamic acid were purchased from Sigma Chemical Co. (St. Louis, MO). NS-398 and nimesulide were obtained from Cayman Chemical Co. (Ann Arbor, MI). (*R*)-Flurbiprofen and (*S*)-flurbiprofen were generously provided by the Upjohn Co. (Kalamazoo, MI). Meclofenamic acid was a generous gift from Parke-Davis and SQ 29,535 from Bristol Myers-Squibb. The selective PGHS-1 and PGHS-2 inhibitors, SC 8076 and SC 8092, were generously provided by Monsanto (St. Louis, MO). Ram seminal vesicles were purchased from Oxford Biomedical Research, Inc. (Oxford, MI). PGHS-1 was purified from ram seminal vesicles as described earlier (Marnett et al., 1984). The protein had a specific activity of 20 μmol of arachidonic acid min^{-1} mg^{-1}, and the percentage of holoenzyme was 18%. ApoPGHS was prepared as described earlier, and holoenzyme contamination was less than 2% in this preparation (Odenwaller et al., 1990). Human PGHS-2 was a generous gift from J. Gierse (Monsanto) and had a specific activity of 14 μmol of arachidonic acid min^{-1} mg^{-1}.

Cyclooxygenase Activity. Oxygen consumption was measured at 37 °C with a Gilson Model 5/6 oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) equipped with a Clark electrode and a thermostatted cuvette. Purified enzyme aliquots (23 μg) were added to 100 mM Tris-HCl at pH 8 containing 500 μM phenol and 1 μM hematin in a final volume of 1.3 mL. Oxygen uptake was initiated by the addition of 100 μM arachidonic acid, and the initial reaction velocity was determined from the linear portion of the oxygen uptake curve.

Comparison of Time-Dependent Inhibition of PGHS-1 to the Induction of Trypsin Protection (Method A). ApoPGHS-1 (2 μM) in 100 mM Tris-HCl at pH 8 was incubated with 1 equiv of inhibitor for 5, 30, 60, or 300 s at 25 °C. Enzyme aliquots were diluted to 0.25 μM and assayed for remaining cyclooxygenase activity by addition to an oxygraph cuvette containing 2 equiv of hematin and arachidonic acid (100 μM) in Tris-HCl buffer. ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of inhibitor for the indicated times and diluted to a 2 μM final concentration on the addition of trypsin (3.1 μM). Proteolytic digestion was performed at 25 °C for 15 s. Trypsinization was quenched by the addition of 1.8 mM PMSF followed by incubation at 4 °C for 5 min.

Reversibility of PGHS Resistance to Trypsin Protection (Method B). ApoPGHS-1 (2.2 μM) in 100 mM Tris-HCl at pH 8 was incubated with 1 equiv of inhibitor at 25 °C for 5 s and diluted to a 2 μM final concentration on addition of trypsin to a final concentration of 3.1 μM. Aliquots were removed at 5, 30, 60, or 300 s, placed into tubes containing PMSF (1.8 mM), and then incubated at 4 °C for 5 min.

Control experiments were performed to confirm that the proteolytic activity of trypsin was unaffected by incubation with inhibitors. A reaction mixture containing trypsin with inhibitor (2 μM) was added to a cuvette containing 0.5 mM TAME in 100 mM Tris-HCl at pH 8. The initial rate of proteolysis was monitored at 247 nm. The rates of proteolysis in the presence or absence of inhibitors were essentially the same.

Gel Electrophoresis. PGHS aliquots (2 μg) were heated at 95 °C for 3 min in SDS sample buffer containing dithiothreitol and were loaded on a 10% polyacrylamide minigel. Electrophoresis was performed according to the procedure of Laemmli (1970). The 70 kDa uncleaved protein band and the 38 and 33 kDa protease-cleaved protein bands were scanned on an E-C Apparatus Model EC910 scanning densitometer and quantitated with Hoeffer GS370 Data System software.

RESULTS

Effect of Heme and Cyclooxygenase Inhibitors on Trypsin Sensitivity of ApoPGHS-1 at Short Incubation Times. Previous studies from our and other laboratories on the induction of trypsin resistance by heme or NSAIDs employed prolonged incubations and catalytic amounts of trypsin, conditions that did not enable us to determine the kinetics of induction of trypsin resistance. Therefore, we modified the trypsin protection assay to increase the rate of tryptic cleavage in the absence of heme or inhibitor (method A). Trypsin (3.1 μM) was added to apoPGHS-1 (2 μM), and reactions were stopped after 15 s by addition of excess PMSF. PMSF was found to be much more effective than peptidic trypsin inhibitors (e.g., soybean trypsin inhibitor). The rapid cleavage of PGHS that occurred under these conditions enabled us to detect protection by heme and NSAIDs at times as short as 5 s after their addition. In a typical experiment, apoPGHS and heme or inhibitor were incubated for varying lengths of time and aliquots were removed for the cyclooxygenase assay and for tryptic cleavage. Previous studies have indicated that reconstitution of apoPGHS-1 with hematin also protects the apoenzyme from trypsin cleavage at Arg^{277} (Chen et al., 1987). The time course for induction of trypsin resistance in apoPGHS-1 reconstituted with hematin was also examined at short

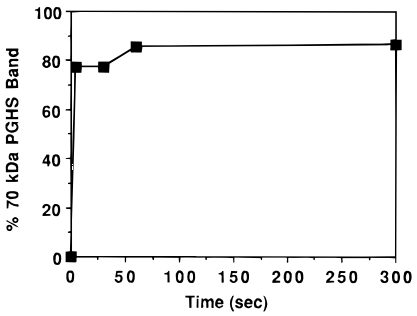

FIGURE 1: Time course of hematin protection of apoPGHS-1 from trypsin cleavage. ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of hematin in DMSO. At the indicated times, 2 μM enzyme aliquots were assayed for the extent of trypsin cleavage (method A).

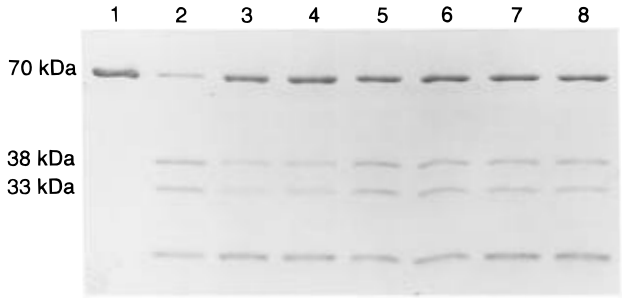

FIGURE 2: Effect of cyclooxygenase inhibitors on trypsin sensitivity of apoPGHS-1 at short times. ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of inhibitor or hematin for 5 s at 25 °C and then treated with trypsin (3.1 μM) for 15 s according to method A: untrypsinized apoPGHS-1 (lane 1), apoPGHS-1 (lane 2), apoPGHS-1 + hematin (lane 3), apoPGHS-1 + SQ 29,535 (lane 4), apoPGHS-1 + ibuprofen (lane 5), apoPGHS-1 + mefenamic acid (lane 6), apoPGHS-1 + meclofenamic acid (lane 7), and apoPGHS-1 + indomethacin (lane 8).

incubation times. Treatment of apoPGHS-1 with 1 equiv of heme for 5 s resulted in 77% protection from trypsin cleavage (Figure 1). To assess the reversibility of heme binding to the apoenzyme, the trypsin protection assay was further modified. ApoPGHS-1 was treated with 1 equiv of hematin for 5 s, and the time period of PGHS digestion with trypsin was varied from 5 to 300 s (method B). When apoPGHS-1 was treated with 1 equiv of the heme prosthetic group, protection against tryptic cleavage persisted such that only a 2% decrease in protection was seen after 300 s of treatment. This result indicates that binding of the heme prosthetic group to the apoenzyme is only very slowly reversible. Figure 2 depicts a gel on which samples of apoprotein incubated with inhibitors for 5 s prior to trypsin addition were analyzed for cleavage at Arg^{277}. Significant protection was afforded by all the inhibitors assayed, indicating that the conformational change leading to trypsin resistance is very rapid.

Attempts to perform similar experiments with PGHS-2 were unsuccessful. ApoPGHS-2 is resistant to cleavage by trypsin in the region equivalent to His^{274}–Gln^{284} but cuts near its C-terminus to produce a protein fragment with a slightly reduced molecular size (Sirois & Richards, 1992). Cleavage of apoPGHS-2 to a slightly smaller fragment was also observed after treatment with chymotrypsin, elastase, and V8 protease. However, SQ 29,535 did not protect apoPGHS-2 from cleavage by trypsin or any of the other proteases. ApoPGHS-2 was resistant to cleavage by thrombin and thermolysin but was extensively degraded by subtilisin, lysyl endopeptidase, and papain.

Comparison of Time-Dependent Cyclooxygenase Inactivation of PGHS with Resistance to Trypsin Cleavage by SQ 29,535. SQ 29,535 is a potent inhibitor of the cyclooxygenase activity of sheep PGHS-1 (Harris et al., 1986; Hall et al., 1986; Pal et al., 1992). Kinetic analysis revealed that SQ 29,535 was a competitive inhibitor of PGHS-1 with a K_i of 4 μM. SQ 29,535 was also a competitive inhibitor of the inducible PGHS-2 enzyme, albeit with a higher K_i value (40 μM). These results suggest that the formation of the initial (E−I) complex (eq 1) between SQ 29,535 and the PGHS protein occurs in the fatty acid access channel of PGHS. This is supported by the inability of SQ 29,535 to inhibit peroxidase activity. SQ 29,535 is also a time-dependent cyclooxygenase inhibitor. The maximal rate constant (k_{inact}) for PGHS inactivation and the apparent concentration (K_I) of inhibitor causing the half-maximal rate of inactivation are shown in Table 1. At stoichiometric concentrations of PGHS-1 or PGHS-2 and SQ 29,535, substantial inhibition of the cyclooxygenase activity occurred within the first minute of incubation. Under these conditions, the peroxidase activity remained unaffected. The cyclooxygenase activity

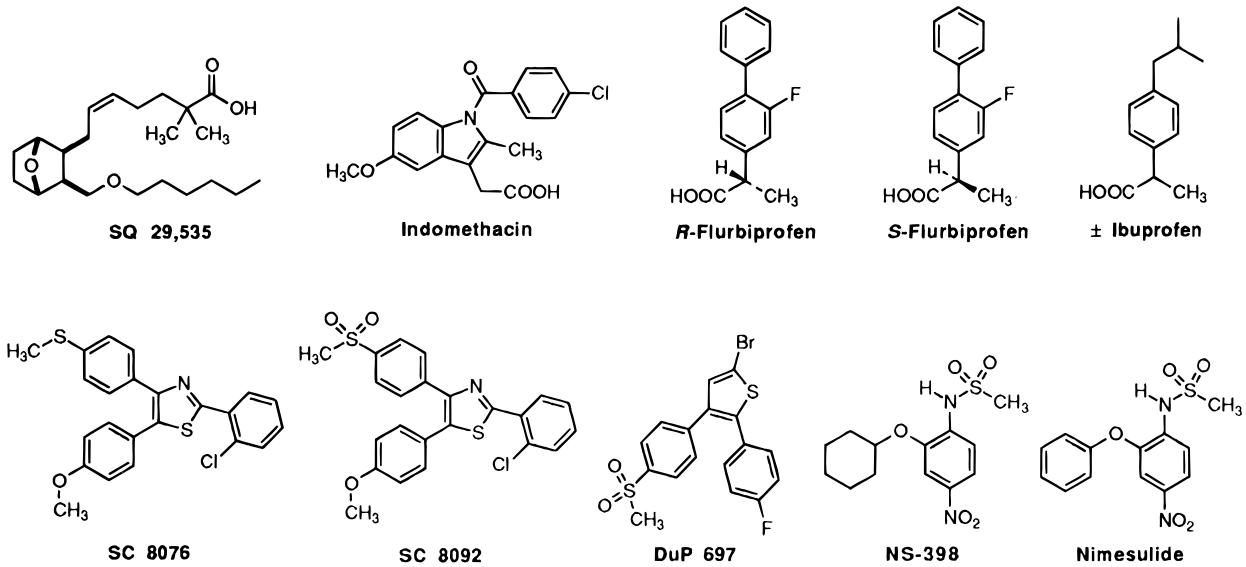

FIGURE 3: Chemical structures of the inhibitors.

Table 1: Kinetic Parameters (k_{inact} and K_I) for Time-Dependent Cyclooxygenase Inhibition by NSAIDs[a]

	cyclooxygenase-1			cyclooxygenase-2		
inhibitor	k_{inact} (s^{-1})	K_I (μM)	k_{inact}/K_I ($s^{-1}\,\mu M^{-1}$)	k_{inact} (s^{-1})	K_I (μM)	k_{inact}/K_I ($s^{-1}\,\mu M^{-1}$)
SQ 29,535	0.8	0.7	1.1	0.9	17	0.05
Indomethacin[b]	0.5	7.2	0.07	–	–	–
Meclofenamic acid[b]	0.1	4.5	0.02	–	–	–
DuP 697[c]	–	–	–	0.02	2.19	0.009
NS-398[c]	–	–	–	0.05	11.5	0.004
SC 8076	0.03	0.2	0.1	–	–	–
SC 8092	–	–	–	0.1	1.0	0.1

[a] None of the compounds tested inhibited peroxidase activity. [b] Tang et al. (1995). [c] PGHS-2 selective inhibitors (Copeland et al., 1994).

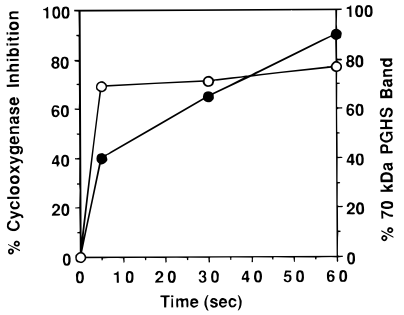

FIGURE 4: Time course of SQ 29,535 protection of apoPGHS-1 from tryptic cleavage and inhibition of cyclooxygenase activity. ApoPGHS-1 (2 μM) was incubated with 1 equiv of SQ 29,535. At the indicated times, enzyme aliquots were diluted to 0.25 μM and assayed for remaining cyclooxygenase activity (closed circles). ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of SQ 29,535 and diluted to a 2 μM final concentration on addition of trypsin. The extent of trypsin cleavage was determined by method A (open circles).

did not return following extensive dialysis of the inhibited enzyme, indicating that inhibition was only very slowly reversible.

To assess whether the conformational change that results in the induction of trypsin resistance correlates to cyclooxygenase inhibition, the time course for the induction of trypsin resistance was compared to the time course for the inhibition of the cyclooxygenase activity. The time courses of induction of resistance to trypsin and inhibition of cyclooxygenase activity by SQ 29,535 were determined by varying the time between addition of the inhibitor to apoprotein and removal of aliquots for both proteolysis and cyclooxygenase assay. Figure 4 demonstrates that induction of resistance to trypsin cleavage was somewhat faster than the loss of enzyme activity.

Resistance of ApoPGHS-1 to Trypsin Cleavage in the Presence of Arachidonic Acid. Agents that protect against tryptic cleavage are believed to bind in the fatty acid binding channel of PGHS. Thus, the ability of arachidonic acid to induce a conformational change in the apoprotein was examined. Incubation of the apoPGHS with increasing concentrations of arachidonic acid afforded increased protection against trypsin, but higher amounts of arachidonic acid were required; the maximal extent of protection was less than that observed with SQ 29,535 (40 versus 73%, respectively) (Figure 5). When the apoprotein was added to an incubation mixture containing 1 equiv of SQ 29,535 and 10 equiv of arachidonic acid, only 41% of the apoprotein was protected from trypsin cleavage (Figure 6). The ability of arachidonic

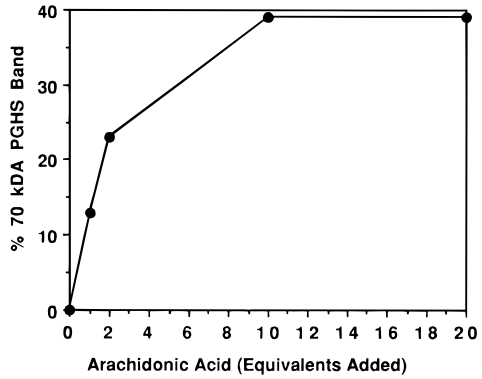

FIGURE 5: Effect of arachidonic acid:PGHS stoichiometry on trypsin cleavage. ApoPGHS-1 (2.2 μM) was incubated with increasing concentrations of arachidonic acid for 5 s at 25 °C. The extent of trypsin cleavage (method A) was compared to that of the untreated protein.

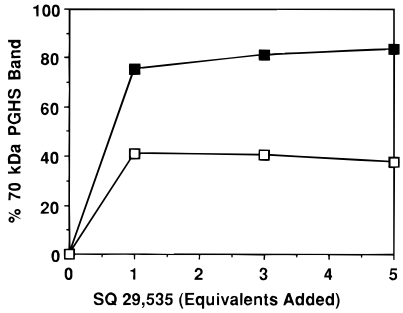

FIGURE 6: Arachidonic acid/SQ 29,535 competition. ApoPGHS-1 (2.2 μM) was added to the reaction tube containing 1, 3, or 5 equiv of SQ 29,535 with or without 10, 30, or 50 equiv of arachidonic acid, respectively, for 5 s at 25 °C. The extent of trypsin cleavage (method A) was compared to that of the untreated protein: SQ 29,535 (closed squares) and SQ 29,535 and arachidonic acid (open squares).

acid to lower the protective effect of SQ 29,535 suggests the two molecules are competing for the same site on the protein and inducing different maximal extents of conformational changes.

Reversibility of Protection against Trypsin Cleavage. Ibuprofen is a reversible, competitive cyclooxygenase inhibitor, so we tested for reversibility in its protection against trypsin. Parallel incubations were conducted with SQ 29,535. When the apoprotein was treated with SQ 29,535, the inhibitor-induced conformational change in the protein was stable such that only a 12% decrease in protection was seen after 300 s of trypsin digestion (Figure 7). In contrast, protection by ibuprofen was reversible by prolonged incubation with trypsin (a 50% decrease in resistance relative to control in approximately 100 s).

Comparison of Time-Dependent Cyclooxygenase Inactivation of PGHS with Resistance to Trypsin Cleavage by Isoform Selective Inhibitors. The recent discovery of PGHS-2 has led to the development of a number of isoform selective inhibitors. Particularly interesting in this regard are the thiazoles SC 8076 and SC 8092. The two molecules differ only in the oxidation state of the sulfur (sulfide versus sulfone); yet SC 8076 is a selective PGHS-1 inhibitor, whereas SC 8092 is a selective PGHS-2 inhibitor. The kinetic parameters (k_{inact} and K_I) for the time- and concentration-dependent inhibition of PGHS-1 and PGHS-2 by SC 8076 and SC 8092, respectively, were determined using the thin layer chromatography assay (Smith & Lands, 1972) and

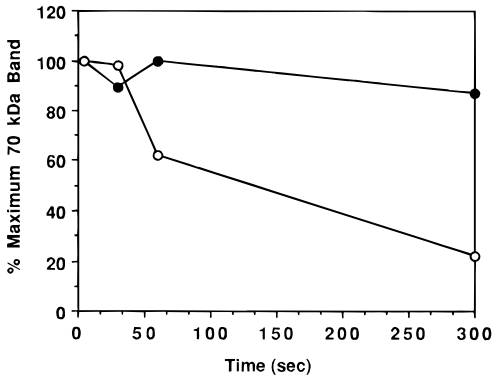

FIGURE 7: Reversibility of trypsin protection by SQ 29,535 and ibuprofen. ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of inhibitor for 5 s at 25 °C. Trypsin (3.1 μM) was added to the incubation mixture for the indicated times. The extent of trypsin cleavage (method B) was determined as described in the Materials and Methods: SQ 29,535 (closed circles) and ibuprofen (open circles). The extent of 70 kDa protein remaining at 5 s was taken to represent the maximum resistance to trypsin cleavage and was normalized to 100%.

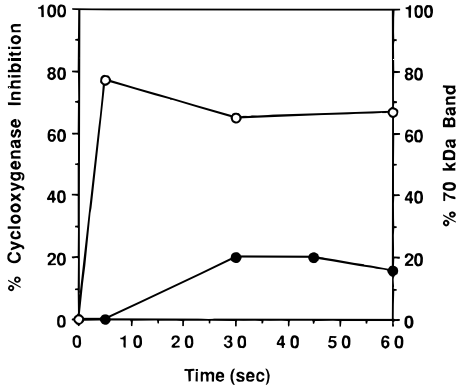

FIGURE 8: Effect of SC 8076 on apoPGHS-1 trypsin resistance and cyclooxygenase inhibition. ApoPGHS-1 (2 μM) was incubated with 1 equiv of SC 8076. At the indicated times, enzyme aliquots were diluted to 0.25 μM and assayed for remaining cyclooxygenase activity (closed circles). ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of SC 8076 and diluted to a 2 μM final concentration with the addition of trypsin. The extent of trypsin cleavage was determined by method A (open circles).

are listed in Table 1. Interestingly, both inhibitors protected apoPGHS-1 from trypsin cleavage. After a 5 s preincubation with the protein, SC 8076 displayed 76% protection (Figure 8) and SC 8092 62% protection (Figure 9). However, the time course for trypsin protection did not correspond to the time course of cyclooxygenase inhibition (see Figures 8 and 9). In fact, although SC 8092 altered the conformation of apoPGHS-1 to confer trypsin protection, it induced no detectable cyclooxygenase inhibition. Reversibility of protection was also examined for SC 8076 and SC 8092. The PGHS-1-specific inhibitor SC 8076 displayed a 22% loss of protection during 300 s of trypsin treatment, whereas the PGHS-2 selective inhibitor SC 8092 displayed a 71% loss (Figure 10). Two other selective PGHS-2 inhibitors, namely NS-398 (Futaki et al., 1994) and nimesulide (Vigdahl & Tukey, 1977), which were capable of protecting PGHS-1 from tryptic cleavage were also tested for reversibility of protection. Under the present assay conditions, NS-398 and nimesulide displayed 88, and 69% losses, respectively, in protection of the apoprotein after 300 s (Figure 10).

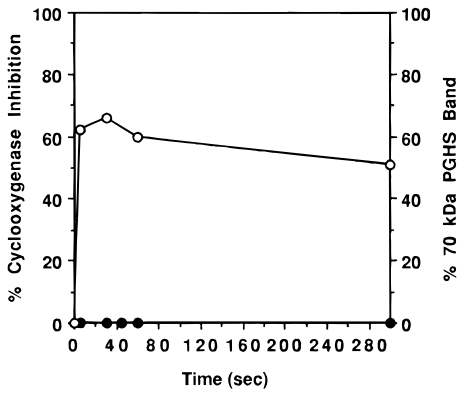

FIGURE 9: Effect of SC 8092 on apoPGHS-1 trypsin resistance and cyclooxygenase inhibition. ApoPGHS-1 (2 μM) was incubated with 1 equiv of SC 8092. At the indicated times, enzyme aliquots were diluted to 0.25 μM and assayed for remaining cyclooxygenase activity (closed circles). ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of SC 8092 and diluted to 2 μM on addition of trypsin. The extent of trypsin cleavage was deteremined by method A (open circles).

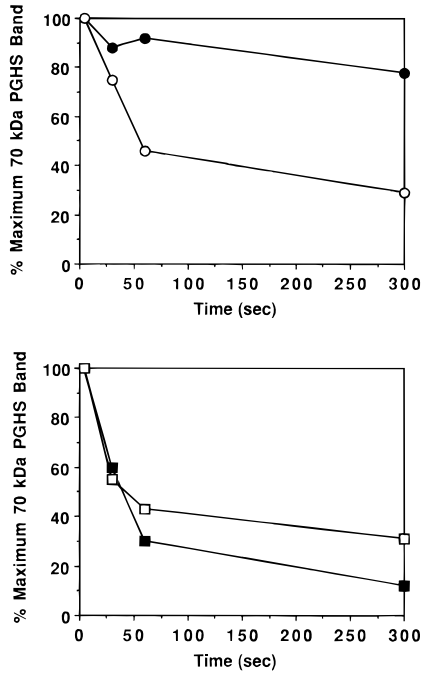

FIGURE 10: Reversibility of trypsin protection by SC 8076, SC 8092, NS-398, and nimesulide. ApoPGHS-1 (2.2 μM) was incubated with 1 equiv of inhibitor for 5 s at 25 °C. Trypsin (3.1 μM) was added to the incubation mixture for the indicated times (method B). Top panel: SC 8076 (closed circles) and SC 8092 (open circles). Bottom panel: NS-398 (closed squares) and nimesulide (open squares). The extent of 70 kDa protein remaining at 5 s was taken to represent the maximum resistance to trypsin cleavage and was normalized to 100%.

DISCUSSION

The present results indicate that a variety of cyclooxygenase inhibitors, including a product analog (SQ 29,535), aryl aliphatic acids (indomethacin, ibuprofen, and flurbiprofen), and thiazoles that do not contain a carboxylic acid (SC 8076 and SC 8092), induce resistance of apoPGHS-1 to trypsin cleavage at Arg^{277}. The observation that PGHS-2-specific inhibitors such as SC 8092, NS-398, and nimesulide protect PGHS-1 from trypsin cleavage suggests that these agents bind to the PGHS-1 protein and induce a rapid conformational change. However, the subsequent loss of trypsin

protection of the apoenzyme in the presence of these agents suggests that the rapidly induced conformational change is reversible due to the dissociation of the PGHS-2-specific inhibitors. Several observations suggest that trypsin resistance results from inhibitor binding in the arachidonic acid access channel. (1) Arachidonic acid partially protects against induction of trypsin resistance. (2) Cyclooxygenase but not peroxidase activity is inhibited following binding of 1 equiv of inhibitor to protein, and (3) the crystal structure of a PGHS-1−flurbiprofen complex reveals the presence of flurbiprofen at the top of the arachidonic acid access channel with its carboxylate complexed to Arg^{120}, a putative arachidonic acid binding residue. Furthermore, induction of trypsin resistance of PGHS-1 by reversible as well as irreversible inhibitors suggests that the conformational change that leads to trypsin resistance results from the initial association of the apoenzyme with the inhibitors.

No cyclooxygenase inhibition was detected for any of the purely competitive PGHS-1 inhibitors or the time-dependent PGHS-2 selective inhibitors because the PGHS-1-inhibitor solutions were diluted 10-fold for the cyclooxygenase assay which reduced the inhibitor concentrations below their IC_{50} values. In contrast, irreversible or slowly reversible inhibitors remained bound to the enzyme after dilution so reduced cyclooxygenase activity was observed. Thus, the protocol for the cyclooxygenase assay that we employed was only capable of detecting tightly bound, slowly reversible inhibitors. With respect to the interaction of PGHS-1 with "selective" PGHS-2 inhibitors, our findings confirm those of Copeland et al. indicating that PGHS-2 selective inhibitors are, in fact, reversible inhibitors of PGHS-1 and their selectivity for PGHS-2 is due to their time-dependent interaction with PGHS-2. Comparable interactions are obtained for PGHS-1 selective inhibitors interacting with PGHS-2.

The ability of SC 8092 and other inhibitors to induce trypsin resistance in apoPGHS-2 could not be monitored due to the resistance of apoPGHS-2 to proteolytic cleavage in the absence of inhibitor. PGHS-2 does not contain Arg at the position corresponding to Arg^{277} of PGHS-1, so it is not cleaved by trypsin into 33 and 38 kDa fragments (Sirois & Richards, 1992). A variety of proteases, which included trypsin, chymotrypsin, elastase, subtilisin, thrombin, lysyl endopeptidase, thermolysin, and V8 protease, were evaluated for their ability to cleave the apoPGHS-2 protein in the region of the protein comparable to PGHS-1. Comparison of the amino acid sequences of the PGHS isozymes in the region of Arg^{277} indicates that there are seven potential cleavage sites in the PGHS-2 isoform. However, none of the proteases tested cleaved apoPGHS-2 into 33 and 38 kDa fragments. Thus, if apoPGHS-2 contains a loop corresponding to the His^{274}−Gln^{284} loop of PGHS-1, it does not appear to be sufficiently exposed to enable proteolysis.

Induction of trypsin resistance is a valuable technique for probing the initial interaction of PGHS-1 with substrates and with reversible and irreversible inhibitors. One potential complication of using this approach to study protein−inhibitor association is that the experiments can only be performed with the apoprotein because the holoprotein is itself resistant to tryptic cleavage. Thus, it is possible that the presence of heme in the holoprotein may alter the interaction of the various inhibitors with the enzyme. This seems unlikely for three reasons. First, an excellent correlation exists between the ability of a diverse series of compounds to induce trypsin resistance and to serve as competitive inhibitors of the cyclooxygenase activity. Second, compounds that are slow, tight-binding inhibitors of cyclooxygenase activity induce resistance to trypsin that is irreversible on the time scale of the experiments, whereas compounds that are purely competitive inhibitors induce resistance that is readily reversible. Thus, the behavior of compounds as reversible or irreversible inducers of trypsin resistance corresponds to their ability to serve as competitive versus slow, tight-binding inhibitors by the classic mechanism described in eq 1. Third, measurements of the quenching of the fluorescence of SC 8076 by the heme prosthetic group of PGHS-1 indicate that this inhibitor and heme bind independently of each other.[2] The close correlation between the ability of a diverse series of compounds to induce trypsin resistance and to competitively inhibit the cyclooxygenase activity suggests that induction of trypsin resistance reflects the initial binding of the inhibitors in the substrate access channel. This binding is rapid and readily reversible.

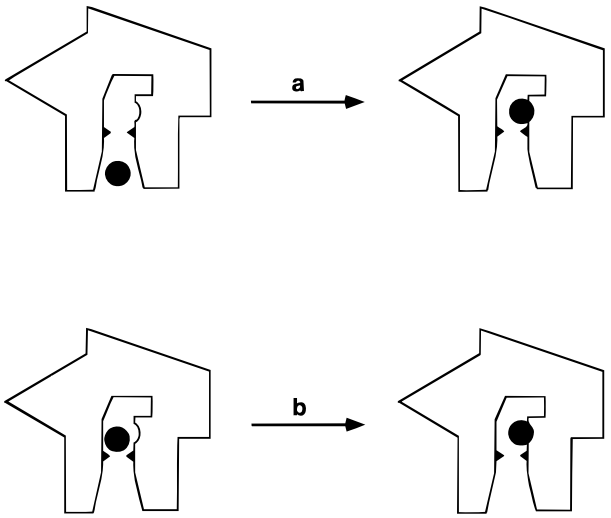

FIGURE 11: Models for PGHS-1 inhibitor interaction.

A subgroup of these compounds forms a tighter complex that is not reversible on the time scale of the trypsin cleavage incubations or on dilution. The rate coefficients for the formation of the tight complexes (k_{inact}) vary significantly with inhibitor. The crystal structure of PGHS-1 exhibits a hydrophobic substrate access channel that is divided into two sections separated by a narrowing formed by the residues Arg^{120} and Tyr^{355}. Arg^{120} also serves as the residue that ion pairs to the carboxylate contained in substrates and inhibitors. The structure of the flurbiprofen−PGHS-1 cocomplex, which presumably represents the tightly bound complex, reveals that flurbiprofen is bound well up in the arachidonic acid binding site past the narrowing at Arg^{120} and Tyr^{355}. Consideration of the present results and the structure of the flurbiprofen−PGHS-1 complex suggests two models of inhibitor−enzyme association leading to reversible and then irreversible binding (Figure 11). In model a, the inhibitor binds at the mouth of the substrate access channel and then moves up the channel past the narrowing at Arg^{120} and Tyr^{355}. Translation up the channel would represent the time-dependent step. In model b, the inhibitor binds in the channel

[2] C. Lanzo, J. Beecham, and L. J. Marnett, manuscript in preparation.

above the narrowing at Arg^{120} and Tyr^{355} and then probes the sides of the channel for stabilizing interactions. The stabilization of the complex would represent the time-dependent step. Neither the present experiments nor the available structures determined by X-ray diffraction enable us to choose between these two mechanisms. This will require kinetic studies of the movement of inhibitors within the channel or X-ray analysis of cocrystals of PGHS-1 or PGHS-2 with isozyme selective inhibitors.

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SUPPORTING INFORMATION AVAILABLE

Figure 1 showing the reversible inhibition of cyclooxygenase activity of PGHS-1 by SQ 29,535, Figure 2 showing the semilogarithmic and double-reciprocal plots for the time- and concentration-dependent inhibition of PGHS-1 by SQ 29,535, Figure 3 showing the semilogarithmic and double-reciprocal plots for the time- and concentration-dependent inhibition of PGHS-1 by SC 8076, Figure 4 showing the semilogarithmic and double-reciprocal plots for the time- and concentration-dependent inhibition of PGHS-2 by SC 8092, Figure 5 showing the effect of (*R*)- and (*S*)-flurbiprofen on PGHS-1 trypsin resistance and cyclooxygenase inhibition, Figure 6 showing the effect of NS-398 and nimesulide on PGHS-1 trypsin resistance and cyclooxygenase inhibition (11 pages). Ordering information is given on any current masthead page.

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