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O-Acetylsalicylhydroxamic Acid, a Novel Acetylating Inhibitor of Prostaglandin H_2 Synthase: Structural and Functional Characterization of Enzyme-Inhibitor Interactions

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ABSTRACT

Aspirin is unique among clinically used nonsteroidal antiinflammatory drugs in that it irreversibly inactivates prostaglandin (PG) H_2 synthase (PGHS) via acetylation of an active-site serine residue. We report the synthesis and characterization of a novel acetylating agent, *O*-acetylsalicylhydroxamic acid (AcSHA), which inhibits PGE_2 synthesis in vivo and blocks the cyclooxygenase activity of PGHS in vitro. AcSHA requires the presence of the active-site residue Ser-529 to be active against human PGHS-1; the S529A mutant is resistant to inactivation by the inhibitor. Analysis of PGHS inactivation by AcSHA, coupled with the X-ray crystal structure of the complex of ovine PGHS-1 with AcSHA, confirms that the inhibitor elicits its effects via acetylation of Ser-529 in the cyclooxygenase active site. The crystal structure reveals an intact inhibitor molecule bound in the enzyme's cyclooxygenase active-site channel, hydrogen bonding with Arg-119 of the enzyme. The structure-activity profile of AcSHA can be rationalized in terms of the crystal structure of the enzyme-ligand complex. AcSHA may prove useful as a lead compound to facilitate the development of new acetylating inhibitors.

Prostaglandin H_2 synthase, the first enzyme in the biotransformation of arachidonic acid to prostaglandins, has been implicated in a variety of disease processes, particularly inflammation (Smith et al., 1996; Garavito and DeWitt, 1999; Marnett et al., 1999). There are two forms of the enzyme: the constitutively expressed PGHS-1, and the inducible PGHS-2. PGHS-1 is found in most tissues, whereas PGHS-2 is found primarily at sites of inflammation, in tumors, and in the central nervous system. Each enzyme isoform has both cyclooxygenase and peroxidase activities. The cyclooxygenase activity is the target for the class of therapeutic agents termed nonsteroidal anti-inflammatory drugs (NSAIDs); the peroxidase activity maps to a spatially distinct active site (Picot et al., 1994) and is unaffected by NSAIDs.

Most NSAIDs, such as aspirin, indomethacin, and ibuprofen, interfere with the binding of arachidonic acid in the cyclooxygenase active site of the enzyme. Aspirin (ASA) and its *O*-(acetoxyphenyl)alkyl sulfide analogs are the only NSAIDs known to irreversibly inhibit PGHS by acetylating an active-site serine residue (residue 529 of human PGHS-1) (Roth et al., 1975, 1983; Kalgutkar et al., 1998). The ability to covalently modify PGHS is the basis for the unique long-lived effect of aspirin on platelet activity, because circulating platelets, unlike most cells, cannot synthesize new PGHS. Acetylation of the active-site serine completely blocks product formation in PGHS-1 but leads to the production of 15(*R*)-hydroxyeicosatetraenoic acid in PGHS-2 (Holtzman et al., 1992; Meade et al., 1993). Ser-529 is not directly involved in catalysis, because mutation of this residue to an alanine has no effect on cyclooxygenase activity except to render the enzyme insensitive to aspirin (DeWitt et al., 1990).

The instantaneous affinity of aspirin for the enzyme is low, with a K_i value of 20 mM (DeWitt et al., 1990). However, this is sufficiently high to ensure that acetylation of serine 529 of PGHS is highly specific. These data suggest that despite aspirin's low affinity for the active pocket of the enzyme, acetylation of the serine progresses rapidly once aspirin has bound in the active site. Once acetylated, the serine side chain is unusually stable to hydrolysis by virtue of its deeply

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ABBREVIATIONS: PGHS, prostaglandin H_2 synthase; NSAID, nonsteroidal antiinflammatory drug; ASA, acetylsalicylic acid (aspirin); AcSHA, acetylsalicylhydroxamic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; DMSO, dimethyl sulfoxide; TRAP, thrombin receptor-activating peptide; PGE_2, prostaglandin E_2; COX, cyclooxygenase; hPGHS, human prostaglandin H_2 synthase.

buried location in the hydrophobic active site, contributing to the specificity.

Aspirin and other NSAIDs do not influence the peroxidase activity of the enzyme. Therefore, after NSAID treatment, the unchecked peroxidase activity can continue to generate free radical species, the biological significance of which is not yet fully understood (Smith and Marnett, 1991). We have synthesized a number of novel acetylated hydroxamic acid derivatives with the hope of producing a dual inhibitor that is capable of cyclooxygenase inhibition via acetylation of the enzyme and peroxidase inhibition via the hydroxamic acid (Davey and Fenna, 1996; Itakura et al., 1997; Henriksen et al., 1998). Although a dual inhibitor has thus far eluded us, we have succeeded in producing a specific acetylating agent, *O*-acetylsalicylhydroxamic acid, in which the hydroxamate group is O-acetylated (AcSHA; Fig. 1). A novel aspect of this compound is the positioning of the acetyl group on the hydroxamic acid rather than on the phenolic hydroxyl, where it is located in aspirin and aspirin analogs. The discovery of a chemically different type of acetylating agent offers scope for the generation of a new family of irreversible inhibitors.

We present here the structural and functional characterization of AcSHA and its interaction with PGHS. Although itself only a modest inhibitor of PGHS, this compound has proven useful as a lead compound, allowing the synthesis of highly potent acetylating agents derived from the salicylhydroxamic acid scaffold (C. M. D., C. T. S., manuscript in preparation). Acetylhydroxamic acids and their metabolites are less acidic than aspirin and thus may lead to PGHS inhibitors having a reduced capacity for topical gastrointestinal injury.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, phosphate-buffered saline, streptomycin, and trypsin were obtained from Invitrogen (Carlsbad, CA). PGHS-1 and arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI). Tris, hematin, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), phenol, hydrogen peroxide, DMSO, indomethacin, salicylhydroxamic acid, salicylic acid, flurbiprofen, A23187, hydrogen peroxide, sodium citrate, ADP, and thrombin receptor-activating peptide (TRAP) were all obtained from Sigma Chemical (St. Louis, MO). *O*-acetylsalicylhydroxamic acid (AcSHA) was prepared as described previously (O'Brien et al., 1997); stock solutions of AcSHA were prepared in absolute ethanol and stored frozen at −20°C.

Preparation of Mutants by Site-Directed Mutagenesis. Mutants of PGHS-1 were prepared using the QuikChange site-directed mutagenesis kit supplied by Stratagene (Cambridge, UK). Starting with pcDNA3-hPGHS-1, which contains a 1.8-kilobase fragment encoding the native human PGHS-1, mutants of human PGHS-1 (S529A and R119Q) were prepared according to instructions supplied by the manufacturer. The oligonucleotide primers used to prepare the mutants were 5′- GGG GCT CCC TTT GCC CTC AAG GGT CTC CTA GG-3′ for S529A and 5′-G CGC CTG GTA CTC ACA GTG CAA TCC AAC CTT ATC CCC-3′ for R119Q. The double-stranded pcDNA3-hPGHS-1 mutant plasmids were sequenced using the dideoxy method to confirm that the mutations were present in the plasmids used for transfections. Plasmids were purified using the Wizard PureFection System from Promega (Madison, WI).

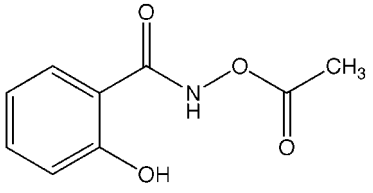

Fig. 1. Structure of AcSHA.

Activity Assays. The enzyme used in the inhibition studies was purified as described previously (Mevkh et al., 1985). Cyclooxygenase activity was measured using a coupled cyclooxygenase-peroxidase assay, monitoring the oxidation of TMPD at 611 nm after the addition of arachidonic acid (Kulmacz and Lands, 1987). Tris-HCl (0.1 M), pH 8.0, was used as the assay buffer; assays contained 40 nM enzyme, 80 μM TMPD, and arachidonic acid concentrations ranging from 5 to 50 μM. Because of enzyme autoinactivation, initial rates were used for all experiments. IC_{50} measurements were carried out by adding known concentrations of AcSHA to aliquots of enzyme and incubating at 37°C for 145 min, followed by rapid cooling on ice and activity measurements. Time-course experiments were carried out by prewarming enzyme solutions to 37°C, adding AcSHA at time 0, and then withdrawing aliquots for assays at various time points. Enzyme activities were normalized to the activity of enzyme solutions containing the vehicle alone. Peroxidase activity was measured spectrophotometrically by using the same assay conditions described above, except that 300 μM H_2O_2 was substituted for arachidonic acid.

Transient Expression of Human PGHS-1 and PGHS-2. Wild-type and mutant pcDNA3-hPGHS constructs were transiently expressed in COS-1 cells. Parental vector pcDNA3 was used as a control for all transfection experiments. COS-1 cells were grown in 2 ml of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 units of penicillin, and 100 μg/ml streptomycin in six-well plates at 37°C and 5% CO_2. Cells were transfected when 50 to 80% confluence was maintained. Purified plasmid DNA (1.5 μg) was transfected into COS-1 cells using 10 μl of LipofectAMINE (Invitrogen) for 24 h as described in manufacturer's protocol.

Assay of Prostaglandins Synthesized by Transfected COS-1 Cells. Forty-eight hours after transfection, the cells were assayed for PGHS activity by analysis of PGE_2 formation. PGE_2 was measured by enzyme immunoassay (R & D Systems Europe, Oxford, UK).

Platelet Aggregation and Thromboxane Production. Platelet aggregation was measured turbidometrically in platelet-rich plasma using a platelet aggregometer (model PAP-4; Biodata Corporation, Horsham, PA). Aliquots of stock solutions of compounds in DMSO were incubated with 495 μl of platelet-rich plasma for 3 min before the addition of the agonist: 1.5 mM arachidonic acid, 10 μM TRAP, 10 μM ADP (high dose), or 2 to 5 μM ADP (low dose). Aliquots (50 μl) were obtained 5 min after the addition of the agonist, added to 1 mM indomethacin, and snap-frozen in liquid nitrogen for analysis of thromboxane B_2 production by enzyme immunoassay.

Crystallization and Data Collection. PGHS-1 was purified from ram seminal vesicles and crystallized essentially as described previously (Picot et al., 1994). Purified enzyme was concentrated to 12 mg/ml and dialyzed overnight versus 20 mM sodium phosphate, pH 6.7, 100 mM NaCl, 0.6% w/w β-octyl glycoside, and 100 μM diethyldithiocarbamate. After dialysis, hanging drop-crystallization experiments were set up at 291°K using 4-μl drops and a reservoir solution of 6.5% polyethylene glycol 4000, 65 mM sodium phosphate, pH 6.7, and 410 mM NaCl. Small rod-like crystals were observed after several weeks; their dimensions were approximately 80 × 80 × 300 μm. At this point the crystallization chamber was opened, 0.1 μl of a 335 mM solution of AcSHA in absolute ethanol was injected into the drop, and the chamber was resealed. After 3 days, the crystals were harvested by a stepwise procedure into a cryoprotectant buffer containing 20 mM sodium phosphate, pH 6.7, 150 M NaCl, 0.4% β-octyl glycoside, 100 μM diethyldithiocarbamate, 2 mM AcSHA, and 30% (v/v) glycerol, and the buffer was flash-cooled in liquid nitrogen. Diffraction data were collected from a single crystal maintained at 100 K in a stream of cold nitrogen gas. A MAR image plate detector

(marUSA Inc., Evanston, IL) mounted on a rotating anode generator was used. DENZO and SCALEPACK (Otwinowski and Minor, 1997) were used for integration and scaling of the intensities. Details of data collection are shown in Table 1.

Crystallographic Refinement. The AcSHA–PGHS-1 crystals are isomorphous with those of the previously solved 3.1-Å structure of the complex of PGHS-1 and flurbiprofen (Garavito et al., 1995). Accordingly, the starting coordinates for the refinement were derived from the flurbiprofen complex. All nonprotein atoms except for the heme atoms were removed from the coordinate file, and this structure was subjected to rigid-body minimization using X-PLOR (Brunger, 1992). At this stage, good electron density was observed in difference maps for the inhibitor and carbohydrate atoms. Refinement continued in CNS version 0.9 (Brunger et al., 1998), using iterative cycles of maximum likelihood refinement, map inspection, and model building. Anisotropic ΔB corrections and appropriate solvent models were calculated by the program. Strict noncrystallographic symmetry restraints and tight geometric and thermal parameter restraints were maintained throughout. The refinement converged at R/R_{free} values of 0.22 and 0.25, respectively. Details of the refinement are given in Table 1. Coordinates and structure factors have been deposited in the Protein Data Bank (accession number 1EBV).

Nomenclature. The numbering of amino acids differs between ovine and human PGHS-1. Crystallographic experiments were performed with the ovine enzyme, whereas functional experiments used both the human and ovine enzymes. To avoid confusion, we use the human numbering scheme throughout this article.

Results

AcSHA Inhibits Product Formation by PGHS-1 and PGHS-2. COS-1 cells were transfected with either a pcDNA3 construct encoding human PGHS or pcDNA3 alone and assayed for PGE_2 production. Sham-transfected cells did not metabolize arachidonic acid, whereas cells expressing PGHS-1 synthesized high levels of PGE_2. Treatment of the cells expressing PGHS-1 with AcSHA or ASA effectively eliminated the synthesis of PGE_2 (Fig. 2A). To investigate whether AcSHA displays isoform selectivity, cells transfected with the PGHS-2 enzyme were examined. Incubation for 45 min with either 500 μM ASA or AcSHA also inhibited PGE_2 production (16,062 ± 2,703 pg/mg to 1,958 ± 177 pg/mg of protein and 4,127 ± 709 pg/mg of protein for ASA and AcSHA, respectively). At 200 μM, AcSHA reduced PGE_2 generation by the two isoforms to the same extent (43.6 ± 3.3% and 50.4 ± 5.5% for PGHS-2 and PGHS-1, respectively), indicating that the compound is not isoform-selective.

TABLE 1
Crystallographic data collection and refinement statistics
R-value = $\Sigma|F_{obs} - F_{calc}|/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors respectively. R_{free} is the cross validation R factor computed for the test set of reflections (7.5% of total reflections were used), which are omitted during the refinement process.

Spacegroup I222	
Unit cell dimensions (Å)	
a	98.9
b	207.5
c	222.0
Data collection statistics	
Resolution (Å)	15.0–3.2
No. observations	124,009
No. unique reflections	35,021
Rmerge	0.113 (overall)
	0.289 (3.2–3.3 Å shell)
Completeness (%)	82.2 (overall)
	75.0 (3.2–3.3 Å shell)
Final refinement parameters	
No. of non-hydrogen atoms[a]	2×4594
Resolution (Å)	25.0–3.20
Working R value	0.218
No. reflections, working set	28,668
Free R value	0.248
No. reflections, test set	2502
rms deviation from ideal geometry of final model	
Bond lengths (Å)	0.008
Bond angles (deg)	1.4
Dihedral angles (deg)	21.7
Improper angles (deg)	0.90

[a] Strict 2-fold noncrystallographic symmetry imposed.

AcSHA Inhibition of Platelet Aggregation. Incubation of platelet-rich plasma with 0.6 to 1.0 mM AcSHA for 30 min inhibited platelet aggregation induced by arachidonic acid to 6.6 ± 0.6% of control values. In contrast, AcSHA did not inhibit platelet aggregation induced by 10 μM TRAP (99 ± 0.6% of control), which is independent of cyclooxygenase activity.

Ser-529 Is Required for AcSHA Inhibition of PGHS-1 Cyclooxygenase Activity. To determine whether PGHS-1 inhibition by AcSHA is dependent on the presence of Ser-529, the inhibitor's activity against the S529A mutant of human PGHS-1 was examined. In COS-1 cells transfected with the S529A mutant, PGE_2 production measured after the addition of 60 μM arachidonic acid was similar to levels found in cells

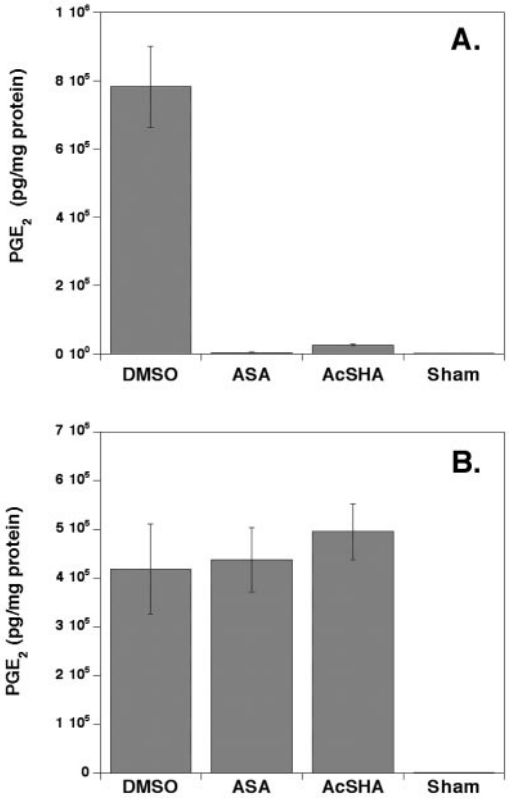

Fig. 2. Inhibition of wild-type and mutant human PGHS-1 by AcSHA and ASA. COS-1 cells were transfected with pcDNA3 constructs encoding human PGHS-1 and with sham constructs of pcDNA3 alone. The cells were assayed for PGE_2 biosynthetic activity 15 min after application of 60 μM arachidonic acid by using an enzyme-linked immunosorbent assay kit. Cells were treated with 500 μM ASA, 500 μM AcSHA, or DMSO vehicle alone. The data represent the mean ± S.E. of three individual experiments, each involving a separate transfection. PGE_2 levels are expressed as pg/mg total protein. A, inhibition of wild-type hPGHS-1. B, inhibition of the S529A mutant of hPGHS-1.

transfected with the native enzyme: 419,000 ± 92,661 pg/mg of protein for S529A compared with 782,030± 118,280 pg/mg of protein for the wild type. However, in contrast to cells transfected with the wild-type enzyme, COS-1 cells transfected with the S529A mutant showed no change in PGE_2 generation after treatment with either 500 μM ASA or 500 μM AcSHA (Fig. 2B).

Role of Arg-119 in the Inhibition of PGHS-1 Activity by AcSHA. To examine the role played by Arg-119 in the inhibition of PGHS-1 cyclooxygenase activity by AcSHA, COS-1 cells transfected with the R119Q mutant of hPGHS-1 were used. Exposure of these cells to 60 μM arachidonic acid produced much lower levels of PGE_2 than in cells transfected with wild-type hPGHS-1: 2,882 ± 354 pg/mg of protein for R119Q versus 782,030 ± 118,280 pg/mg of protein for wild-type. Preincubation of cells expressing the R119Q mutant with 500 μM AcSHA or 500 μM ASA had little or no effect on these residual levels of PGHS activity: 3342 ± 761 pg of PGE_2 were produced per mg of protein for AcSHA-treated cells and 1,902 ± 108 pg/mg for ASA-treated cells.

PGHS-1 Inhibition by AcSHA In Vitro. When preincubated with the enzyme before assay, AcSHA inhibits the cyclooxygenase activity of purified ovine PGHS-1 in a dose-dependent manner, with an IC_{50} value of approximately 4.5 mM (Fig. 3). However, in the absence of preincubation, no instantaneous inhibition is seen at AcSHA concentrations as high as 10 mM. The time dependence of cyclooxygenase inhibition was measured at 37°C, and the rate at which enzyme activity was lost was observed to follow first-order kinetics (Fig. 4A). AcSHA had neither an instantaneous nor a time-dependent effect on the peroxidase activity of the enzyme. Salicylhydroxamic acid, the hydrolysis product of AcSHA, exerted no time-dependent inhibitory effect on the cyclooxygenase activity of the enzyme. Once inactivated, PGHS did not recover catalytic activity even after prolonged incubation in inhibitor-free solution (data not shown). The addition of ibuprofen, a competitive inhibitor known to bind in the cyclooxygenase active site (Selinsky et al., 2001), reduced the rate at which AcSHA inactivated PGHS (Fig. 4B). Taken together, these data are consistent with a model in which AcSHA acts as an affinity label of PGHS, inactivating the cyclooxygenase activity in the same manner as does ASA, via an irreversible acetylation event in the enzyme active site.

The simplest model that describes the interaction of affinity labels such as ASA with their target enzyme assumes that the inhibitor first associates reversibly with the enzyme, and then covalently modifies it: $E + I \leftrightarrow EI \rightarrow EI^*$, where EI* denotes the irreversibly modified enzyme. The kinetics of AcSHA inhibition are not consistent with this simple model but rather show signs of substantial positive cooperation (Fig. 5). This leads to rates of enzyme inactivation that are slow at low AcSHA concentrations but increase significantly when the inhibitor concentration exceeds ~3 mM. The sig-

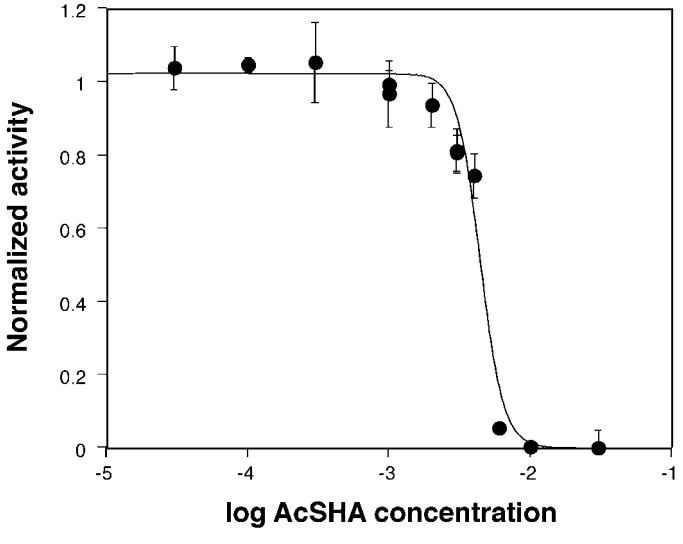

Fig. 3. Inhibition of ovine PGHS-1 in vitro. Purified enzyme was incubated with indicated concentrations of inhibitor at 37°C for 145 min and then cooled on ice and immediately assayed for activity. Enzyme activity is represented as a normalized value, obtained by dividing the observed activity by the activity of the untreated enzyme. Error bars represent the S.E. of three to five independent measurements.

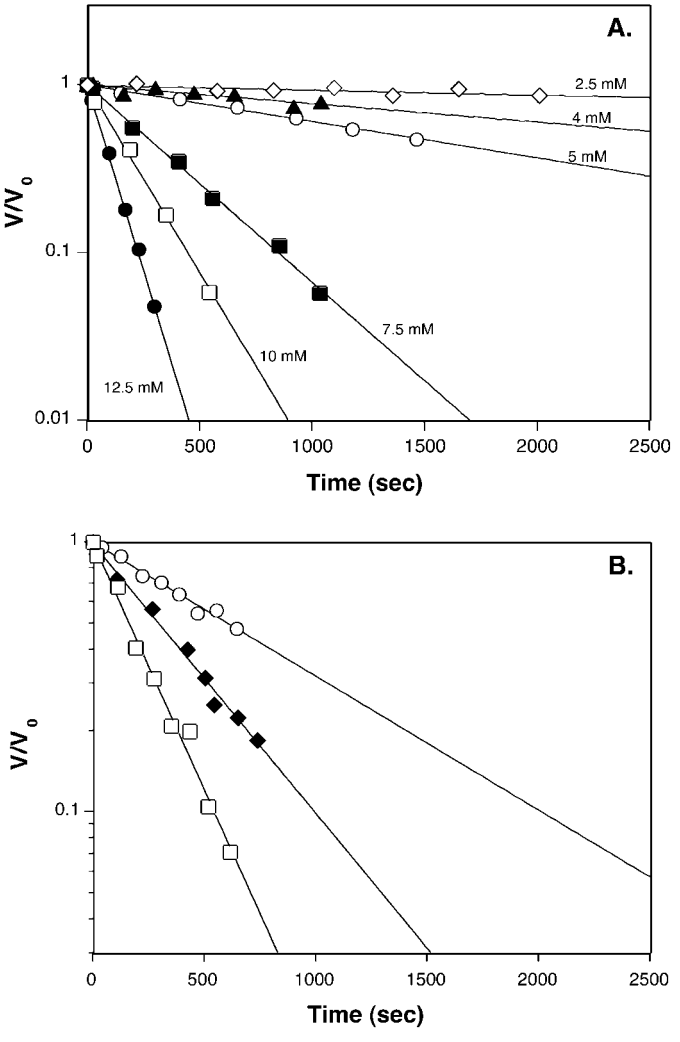

Fig. 4. Cyclooxygenase inhibition by AcSHA is time-dependent. Purified ovine PGHS-1 was incubated with varying concentrations of inhibitor at 37°C; aliquots were withdrawn at different times and assayed for activity. Enzyme activity is plotted as the normalized activity V/V_0, where V = enzyme activity at time t and V_0 = enzyme activity at time 0. A, representative time course data showing the variation of inhibition with AcSHA concentration. The lines represent least-squares fits to the data; each line is identified by the appropriate inhibitor concentration. B, antagonism of AcSHA inhibition by ibuprofen. Enzyme was incubated with 10 mM AcSHA and either 0, 10, or 50 μM ibuprofen [the K_i value for ibuprofen is approximately 5 μM (Mantri and Witiak, 1994)]. □, no ibuprofen; ♦, 10 μM ibuprofen; ○, 50 μM ibuprofen.

moidal dependence of k_{obs} (the observed rate constant for enzyme inactivation) on inhibitor concentration is consistently observed with different enzyme preparations and when using different detergents (data not shown). The data in Fig. 5 are well fit by a Hill coefficient of 3, but the precise significance of this number is unclear, given that PGHS is dimeric.

X-Ray Crystal Structure: Acetylation of the Active-Site Serine. AcSHA blocks PGE_2 synthesis in vivo and inhibits the cyclooxygenase activity of PGHS-1 in vitro. To understand the mechanism by which AcSHA achieves its effects, an X-ray crystal structure was determined for ovine PGHS-1 treated with the inhibitor. Clear electron density for an acetyl group was observed on the side chain of Ser-529, proving that AcSHA is an acetylating inhibitor. No significant differences were observed between the structures of the enzymes acetylated by AcSHA and ASA (Loll et al., 1995). In both cases, the acetyl group projects into the active-site channel immediately below Tyr-384, the presumptive active-site residue responsible for attacking the arachidonic acid substrate (Figs. 6 and 7). The effect of introducing the acetyl adduct is to close off the upper part of the cyclooxygenase active-site channel and block the access of substrate to Tyr-384. As is the case with the ASA-inhibited enzyme, an apparent hydrogen bond is observed between the carbonyl oxygen of the acetyl adduct and the phenolic hydroxyl group of Tyr-384. The hydrogen bond seems to stabilize the observed conformation of the acetyl group, keeping it in the center of the channel and preventing its folding back out of the way of the incoming substrate. There is no significant difference between the conformation of the enzyme's polypeptide chain between the ASA-inhibited structure, the AcSHA-inhibited structure, and the flurbiprofen-inhibited structure: The root-mean-square difference in α carbon positions between the AcSHA structure and the ASA structure is 0.31 Å and between the AcSHA structure and the flurbiprofen structure is 0.26 Å.

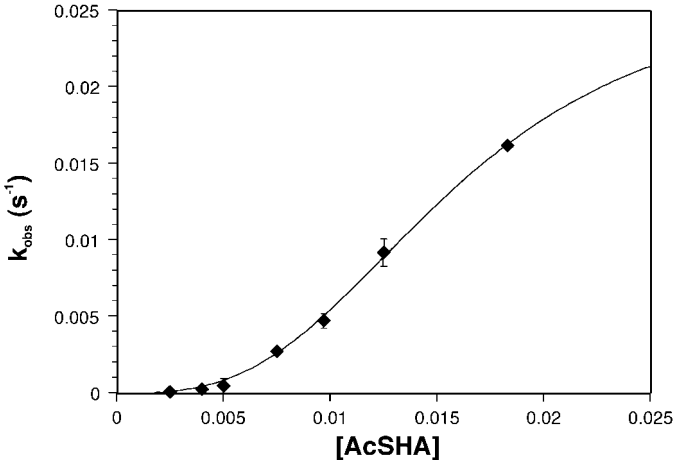

Fig. 5. Evidence for positive cooperativity in PGHS-1 inhibition by AcSHA. The first-order rate constant for enzyme inactivation, k_{obs}, was obtained from time-course experiments such as those shown in Fig. 4A. Error bars represent S.E. from three independent determinations. The curve represents a nonlinear least-squares fit to the data of the generalized Hill equation: inactivation rate = $A[AcSHA]^{nH}/(B + [AcSHA]^{nH})$, where A and B are constants and n_H is the Hill coefficient. Values obtained are $A = 0.027\ s^{-1}$, $B = 4 \times 10^{-6}\ M^{nH}$, and $n_H = 2.98$. Residual and χ^2 values for this fit are 0.999 and 3.7×10^{-7}, respectively.

X-Ray Crystal Structure: Binding of AcSHA. A molecule of AcSHA is observed to bind in the cyclooxygenase active site, directly beneath the acetylated side chain of Ser-529 and the side chain of Tyr-384 (Figs. 6 and 7). The inhibitor is essentially completely shielded from solvent. Its aryl ring lies uppermost in the pocket in a hydrophobic portion of the channel formed by the side chains of Val-348, Leu-351, Phe-517, and Ile-522. The hydroxamate and acetyl groups extend downward from here through the channel, with the acetyl group reaching a point just above the constriction in the channel that is formed by Tyr-354 and Arg-119. The hydroxamic acid species is expected to be largely deprotonated at pH 6.7, where the crystals were grown,[1] and hence can form an ion-pair with Arg-119. In addition, the carbonyl oxygen of the acetyl group seems to form a hydrogen bond with the guanidinium group of Arg-119. Apart from this, we observed no specific polar interactions between the inhibitor and the protein. However, the degree of steric complementarity between inhibitor and protein is quite good, so that either the AcSHA molecule or the acetyl group attached to Ser-529 essentially occupies the entire volume of the channel from Arg-119 to Tyr-384. This particular binding site seems to be universally used by NSAIDs of diverse structures. Hence, there is substantial overlap between the volume occupied by AcSHA and those volumes occupied by flurbiprofen, suprofen, indomethacin, SC-558, RS104897, and RS57067 bound to PGHS-1 and/or PGHS-2 (Kurumbail et al., 1996; Loll et al., 1996; Luong et al., 1996).

Discussion

The acetylation of PGH synthase is a useful pharmacological tool, because it gives rise to irreversible inhibition of the enzyme and a situation in which the recovery of PGHS activity depends on the synthesis of new enzyme. To date, the only acetylating compound available for clinical use is aspirin, which is relatively weak and must be given in high doses to achieve anti-inflammatory activity. In addition, aspirin and its metabolites act as topical irritants in the gastrointestinal tract (Hudson et al., 1992). We present a novel acetylating compound, AcSHA, and we show that it accomplishes the same modification of the enzyme as aspirin does: the acetylation of Ser-529.

The precise mechanism by which AcSHA acetylates Ser-529 cannot be demonstrated by a static crystal structure. However, it is expected to involve an initial binding event at the site observed in this structure, possibly mediated by an interaction between the anionic *O*-acetylsalicylhydroxamate group and Arg-119; once it has arrived at this site, the inhibitor can then explore other portions of the COX channel, diffusing upward into the space under Tyr-384 and Trp-386. This upward motion will bring the acetyl group into close proximity with Ser-529, allowing acetylation to occur. Even modest binding in the COX site should give rise to a high local concentration of the acetylating agent near Ser-529, which would be sufficient to ensure efficient inactivation of the enzyme.

The presence of both the acetylated Ser-529 and an intact

[1] Relevant pK_a values are as follows: *O*-acetylsalicylhydroxamic acid, 5.8 (—CONH group); salicylhydroxamic acid, 7.4 (hydroxamic acid) and 9.8 (phenol); salicylic acid, 3.0 (carboxylic acid) and 13.4 (phenol). All valves in water, 298° K, ionic strength, 0.2 M.

inhibitor in our crystal structure allows us to confirm the specific chemical nature of the modification induced by AcSHA in the enzyme and to examine the structural determinants of inhibitor binding. Binding of an inhibitor by an enzyme containing an acetylated serine is not unprecedented; treatment of PGHS-1 with an aspirin analog gives rapid acetylation of Ser-529 and retention of the salicylate-leaving group, which is bound in approximately the same position as AcSHA (Loll et al., 1995). The current structure differs in one significant regard: AcSHA is bound in the COX site and not the salicylhydroxamic acid-leaving group. This can be explained by AcSHA's higher resistance to hydrolysis. Thus, when crystals of PGHS are exposed to a solution of ASA, Ser-529 will be rapidly acetylated, producing negatively charged salicylate as a byproduct. This salicylate binds closely to the positively charged Arg-119 in the COX site, but it can also diffuse away from the enzyme. However, additional salicylate will be produced by nonenzymatic hydrolysis of ASA in the aqueous buffer, ensuring high occupancy of the salicylate binding site on the enzyme. In contrast, after AcSHA acetylates Ser-529, if the resulting salicylhydroxamic acid, which is uncharged at neutral pH, diffuses out of the COX site, it is likely to be replaced by intact AcSHA from solution.

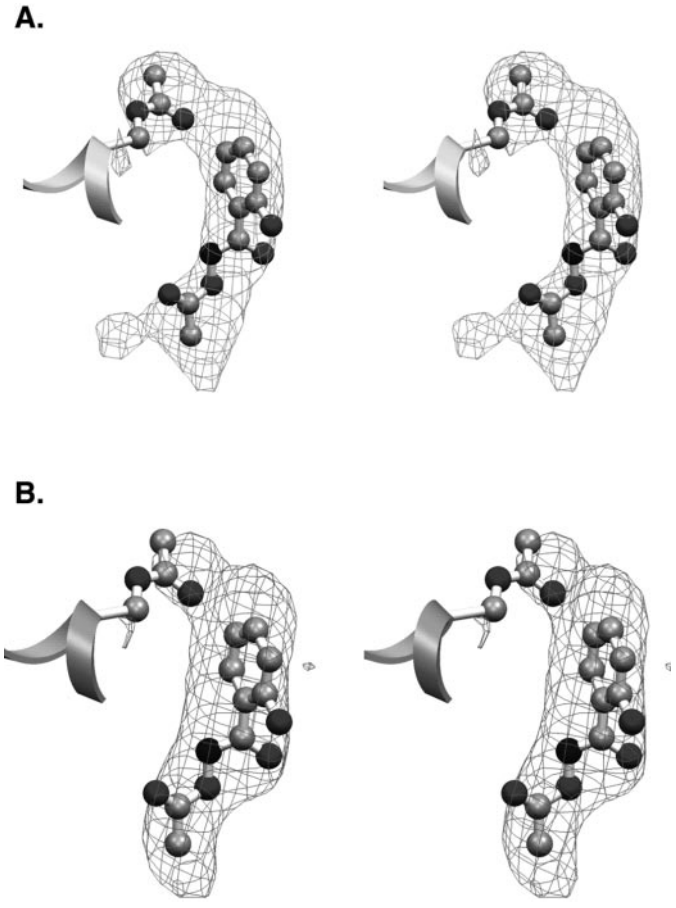

Fig. 6. Stereo images of simulated annealing omit maps showing the electron density for the acetylated Ser-529 side chain and the AcSHA inhibitor. A and B show the electron density for the crystallographically independent A and B protein chains found in the crystal asymmetric unit. The program Setor was used to generate the images (Evans, 1993). Electron density is contoured at the 4σ level.

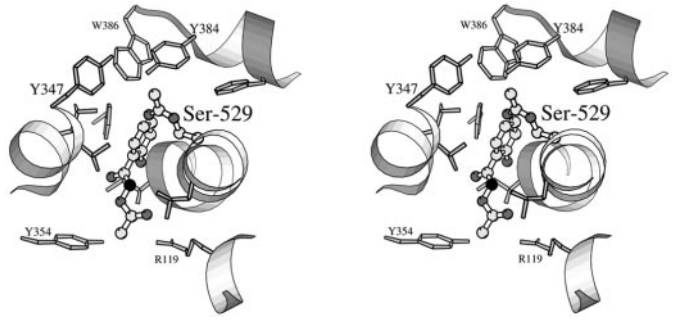

Fig. 7. Stereo view of the binding of the AcSHA inhibitor in the PGHS-1 cyclooxygenase active site. The acetylated side chain of Ser-529 is seen at upper right in ball-and-stick representation, and the AcSHA molecule is shown at center, also in ball-and-stick representation. Carbon atoms are colored light gray, oxygen atoms dark gray, and the nitrogen atom black. Protein side chains near the inhibitor are shown as sticks. Some side chains that are found near the inhibitor are not labeled for the sake of clarity; these include Leu-530 and Val-348 (right and left foreground, respectively), Ile-522 (directly behind AcSHA), and Leu-351 and Phe-517 (upper left, background). The program MOLSCRIPT was used to generate the images (Kraulis, 1991).

The atomic model of the AcSHA–PGHS-1 complex allows for the interpretation of preliminary structure-activity studies in a structural context. Thus, for example, removal or movement to other positions on the ring of the inhibitor's phenolic hydroxyl abolishes the COX inhibitory activity (data not shown). The structure shows that this hydroxyl is not engaged in any hydrogen bonds with the acetylated enzyme; however, it is clear that in the absence of this hydroxyl, the inhibitor cannot entirely fill the available space in the channel, resulting in the loss of van der Waals interactions. Movement of the hydroxyl to the *meta* position would result in a steric clash with the backbone carbonyl oxygen of residue 521 in one orientation of the ring; rotating the ring by 180° would eliminate this clash, but it would place the phenolic hydroxyl in a very favorable position to hydrogen bond with the hydroxyl group of Tyr-384. Such a hydrogen bond would presumably prevent the inhibitor from moving upward in the cyclooxygenase pocket; as discussed above, this upward motion would be required to affect acetylation of Ser-529. A similar argument suggests why a *para* hydroxyl group will not work: such a substituent would pack against the underside of Tyr-384 and would probably prevent upward movement and acetylation. The possibility that Tyr-384 might play an active role in positioning the inhibitor before acetylation is consistent with the observation that this residue is required for efficient acetylation of the enzyme by ASA (Hochgesang et al., 2000).

These results do not preclude a possible reaction mechanism in which the active species for enzyme acetylation is one in which the acetyl group has been transferred from the hydroxamic acid to the serine residue via the phenolic group.[2] We observe no evidence for migration of the acetyl group in solution, but we cannot rule out the formation of very low (but still kinetically significant) levels of the O_{phenol}-acetylated species, either in solution or at the active site of the enzyme. Indeed, such a mechanism could explain the sigmoidal dependence of the acetylation rate on AcSHA concentration (Fig. 5). However, given that formation of the seven-membered ring intermediate that would be required

[2] We acknowledge one of the referees for pointing out this possibility.

for an acetyl transfer is expected to be unfavorable, the likelihood of such a mechanism is currently unclear.

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