O-Acetylsalicylhydroxamic Acid, a Novel Acetyling Inhibitor of Prostaglandin H₂ 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) H₂ synthase (PGHS) via acetylation of an active-site serine residue. We report the synthesis and characterization of a novel acetyling agent, O-acetylsalicylhydroxamic acid (AcSHA), which inhibits PGE₂ 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 acetyling inhibitors.

Prostaglandin H₂ synthase, the first enzyme in the bio-transformation 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 acetyling 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 Kᵣ 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

ABBREVIATIONS: PGHS, prostaglandin H₂ synthase; NSAID, nonsteroidal antiinflammatory drug; ASA, acetylsalicylic acid (aspirin); AcSHA, acetylsalicylhydroxamic acid; TMPD, N,N',N''-tetramethyl-p-phenylenediamine dihydrochloride; DMSO, dimethyl sulfoxide; TRAP, thrombin receptor-activating peptide; PGE₂, prostaglandin E₂; COX, cyclooxygenase; hPGHS, human prostaglandin H₂ 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). Acetylsalicylic 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 tryptophan were obtained from Invitrogen (Carlsbad, CA). PGHS-1 and PGHS-2 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, furibiporgen, 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 QuickChange 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’-GOG CTC CCC TTT GCC TTC AAG GGT CTC CTA GG-3’ for S529A and 5’-G GC C TG CTG GTA CTC AGT G CA A TTC 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).

**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 mM 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. IC50 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 H2O2 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% CO2. 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 PGE2 formation. PGE2 was measured by enzyme immunoassay (R & D Systems Europe, Oxford, UK).

**Platelet Aggregation and Thromboxane Production.** Platelet aggregation was measured turbidimetrically 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 B2 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 mM diethyldithiocarbamate. After dialysis, hanging drop-crystallization experiments were set up at 291°C 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 x 80 x 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 mM 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
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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 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.

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 expressing wild-type 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.

TABLE 1
Crystallographic data collection and refinement statistics

| No. of non-hydrogen atoms$^a$ | 2x4594 |
| Resolution (Å) | 25.0–3.2 |
| 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.
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 PGE2 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 PGE2 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 PGE2 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 IC50 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 ↔ EI → 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.

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.
moidal dependence of $k_{\text{obs}}$ (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. AcSHA structure and the ASA structure is 0.31 Å and be-

![Image](image-url)

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

**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 gastrointes-

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1 Relevant $p_K_a$ values are as follows: O-acetylsalicylhydroxamic acid, 5.8 (COOH group); salicylhydroxamic acid, 7.4 (hydroxamic acid) and 9.8 (phenol); salicylic acid, 3.0 (carboxylic acid) and 13.4 (phenol). All values 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.

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. 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

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$^2$ 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.

Acknowledgments

We gratefully acknowledge Kushol Gupta (University of Pennsylvania School of Medicine, Philadelphia, PA) for providing purified ovine PGHS-1.

References


