Aspirin Inhibition and Acetylation of the Plant Cytochrome P450, Allene Oxide Synthase, Resembles that of Animal Prostaglandin Endoperoxide H Synthase*

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The enzymatic reactions leading to octadecanoid lipid signaling intermediates in plants are similar to those of animals and are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) such as salicylic acid and aspirin. In animals, NSAIDs inhibit the cyclooxygenase (COX) activity of prostaglandin endoperoxide H synthase, which ultimately blocks the formation of prostaglandins. In plants, NSAIDs block the formation of 12-oxo-phytodienoic acid and jasmonates, which are the equivalent signaling compounds. In this study we show that NSAIDs act as competitive inhibitors of allene oxide synthase (AOS), the cytochrome P450 that initiates plant oxylipin synthesis. We also show that aspirin causes the time-dependent inhibition and acetylation of AOS, which leads to the irreversible inactivation of this enzyme. This inhibition and acetylation superficially resembles that observed for the inactivation of COX in animals. In AOS, aspirin acetylates three serine residues near the C-terminal region that appear to be highly conserved among AOS sequences from other plants but are not conserved among “classical” type P450s. The role of these serine residues is unclear. Unlike animal COX, where acetylation of a single serine residue within the substrate channel leads to inactivation of prostaglandin endoperoxide H synthase, the three serine residues in AOS are not thought to line the putative substrate channel. Thus, inhibition by aspirin may be by a different mechanism. It is possible that aspirin and related NSAIDs could inhibit other P450s that have motifs similar to AOS and consequently serve as potential biochemical targets for this class of drugs.

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The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drugs; PGHS, prostaglandin endoperoxide H synthase; AOS, allene oxide synthase; PDA, 12-oxo-phytodienoic acid; JA, 7-iso-jasmonic acid; SA, salicylic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; bkg, background; HPL, hydroperoxide lyase; 13S-HPODE, 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid; 15S-HPETE, 15(S)-hydroperoxy-5(Z),8(Z),11(Z),13(E)-eicosatetraenoic acid.
10) in animals and 12-oxo-PDA, JA, methyl JA, and its derivatives (8, 10, 18–21) in plants. These pentacyclic derivatives then activate numerous physiological roles in their respective organism.

Earlier work indicated that exogenous applications of aspirin and SA blocked the wound response of plants (22). Salicylates were also shown to inhibit JA formation and the JA-induced expression of defense-related genes in plants (23, 24). Because AOS was vital for JA biosynthesis, it was proposed that aspirin and SA acted at this step of the pathway (23). However, reduced levels of JA could also have occurred if allene oxide cyclase activity was inhibited. Thus, the enzymatic step affected by NSAIDs on plant oxylipins was unclear.

To characterize these possible effects of SA and aspirin, we used recombinant AOS prepared from a complementary DNA to AOS from the desert guayule plant (25). This recombinant enzyme was produced in high yields in Escherichia coli using the pGEX-KG expression vector and showed high specific activity for three different fatty acid hydroperoxide substrates. The enzyme was also inhibited by a number of NSAIDs including SA and aspirin. Moreover, aspirin led to the time-dependent, irreversible inhibition of AOS that was similar to its effect on animal cyclooxygenase and also caused the acetylation of three serine residues in the C-terminal region of AOS. This acetylation may have been responsible for the observed irreversible inhibition of AOS.

**EXPERIMENTAL PROCEDURES**

**Construction of pGEX-AOS Vector and Expression of AOS in E. coli**—The EcoRI-XhoI fragment of pRPP30 containing the AOS open reading frame (25) was regenerated by polymerase chain reaction using primers that contained an NcoI restriction site in the sense direction (5′-GCGCCATGCGACCATGCTCTAAACCCC-3′) and a SacI site in the antisense direction (5′-CGCGGATCCTATATACTAGCTCTCTTCAGAAGCG-3′). The polymerase chain reaction product was digested with NcoI and SacI and ligated into the pGEX-KG (26) prokaryotic expression vector to generate the pGEX-AOS expression plasmid. Sequencing verified that no unwanted changes had been introduced by the polymerase chain reactions. The pGEX-AOS plasmid (Fig. 2A) was used to transform E. coli strain JM105, which became the source of recombinant AOS for enzyme studies. Recombinant AOS protein was purified from cultures of JM105/pGEX-AOS using previous methods (27) and analyzed on a 10% SDS-PAGE gel (28) stained with Coomassie Brilliant Blue R (Fig. 2B). Heme staining of a companion gel indicated that heme was only associated with the 53 kDa band (data not shown).

**Enzymatic Analysis of Recombinant AOS**—Lineweaver-Burk analyses of recombinant AOS were performed on reactions that were run in a final volume of 0.5 ml in 50 mM potassium phosphate, pH 8.0, using specialized quartz cuvettes having a 1-mm path length. The fatty acid hydroperoxide substrates used for the reactions, 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid (13S-HPODE), 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid (13S-HPOTE), and 15(S)-hydroperoxy-9(Z),11(Z),13(E)-eicosatetraenoic acid (15S-HPETE), were prepared and purified according to Gardner et al. (29). Reactions were initiated by adding a known quantity of pure AOS in 2 ml of 0.1 M 13S-HPODE. Data was collected for 4 replicates ± S.D. When aspirin was incubated with AOS for 3 min before adding the 13S-HPODE substrate, Lineweaver-Burk analysis initially indicated that it acted as a noncompetitive inhibitor with a Kᵢ of 11 µM. In a separate experiment,
purified AOS (40 ng) was preincubated with 1 ml of 10 mM aspirin for 0, 1, 2, 5, and 10 min and then was combined with 1 ml of 0.1 mM 13\textsuperscript{S}-HPODE in a reaction chamber and assayed for activity. These were compared with preparations where aspirin was omitted. Decay of AOS activity by aspirin showed that it acted as a competitive inhibitor, which irreversibly inhibited activity after several min. In a separate experiment, radioactively labeled aspirin [acetyl-1-14C]acetylsalicylic acid (55 mCi/mmol, American Radiochemical) was used (see below), and samples of 14C-labeled AOS were removed after 0, 1, 2, 5, and 10 min and counted by liquid scintillation to determine whether acetylation of AOS by aspirin coincided with the observed loss of enzyme activity.

Aspirin Acetylation and Analysis of AOS—To test for the ability of aspirin to acetylate AOS, acetylation reactions were performed by a modified procedure (30) using [acetyl-1-14C]acetylsalicylic acid (55 mCi/mmol, American Radiochemical). Thirty mg of purified AOS were incubated with 1 mM 14C-aspirin in a volume of 180 ml. Samples of 60 ml each were removed after 0-, 1-, and 2-h incubations at 37 °C and terminated by adding 600 ml of ice-cold acetone. The precipitate was collected by centrifugation and washed three times with acetone. The protein pellet then was dried under reduced pressure and suspended in loading buffer and subjected to SDS-PAGE. After electrophoresis, the gel was dried, sprayed with EN3HANCETM (NEN Life Science Products) and exposed to x-ray film for 50 days at 280 °C before development. The 53-kDa AOS band was specifically labeled after a 1- or 2-h incubation with 14C-aspirin.

To identify the amino acid residues that were acetylated, 14C-aspirin-labeled enzyme was digested with Lys-C proteinase (Promega) using a protein to protease ratio of 10:1 in the presence of 50 mM ammonium bicarbonate buffer, pH 7.8, containing 0.05% SDS for 4 h at 37 °C. After lyophilization, the resulting endoproteinase digests were analyzed by reverse phase HPLC using a micro Bond Pak C18 column and a linear gradient (0 to 50% B) of solvents A (0.1% trifluoroacetic acid in water) and B (0.08% trifluoroacetic acid in acetonitrile). Absorbance of peaks were monitored at 214 nm, peaks were collected manually, and radioactivity was determined by scintillation counting. Radiolabeled peptides were sequenced by Edman degradation using an Applied Biosystems model 477A equipped with an on-line phenylthiohydantoin-amino acid analyzer. Samples (30%) of the phenylthiohydantoin derivatives released after each Edman cycle were measured for radioactivity by liquid scintillation counting.

RESULTS

Purified, recombinant AOS from E. coli exhibited high turnover rates with each of three different substrates used (Fig. 3). Lineweaver-Burk analysis yielded \( k_{cat} \) values of 4700 s\(^{-1} \) for linoleic hydroperoxide (13S-HPODE), 3700 s\(^{-1} \) for linolenic hydroperoxide (13S-HPOTE), and 1400 s\(^{-1} \) for arachidonic hydroperoxide (15S-HPETE) (Fig. 3). AOS also showed higher affinity (\( K_m = 27 \mu M \)) for 15S-HPETE than it did for 13S-
HPODE ($K_m = 75 \mu M$) or 13S-HPOTE ($K_m = 59 \mu M$) (Fig. 3).

Inhibitor studies using 13S-HPODE as the substrate showed that SA was a competitive inhibitor of AOS with a $K_i$ of 238 $\mu M$ (Fig. 4A). Other NSAIDs such as indomethacin, ibuprofen, and piroxicam yielded similar results (Fig. 4B). When aspirin was incubated with the enzyme for more than 3 min, it appeared to act as a noncompetitive inhibitor of AOS, with a $K_i$ of 11 $\mu M$. D. purified AOS (40 ng) was preincubated with 1 ml of 10 $\mu M$ aspirin for 0, 1, 2, 5, and 10 min and then combined with 1 ml of 0.1 mM 13S-HPODE in a reaction chamber and assayed for activity (filled circle) compared with preparations where aspirin was omitted (open circle). Decay of AOS activity by aspirin showed that it acted as a competitive inhibitor that irreversibly blocked activity after several min. During the same time period, samples of $^{14}C$-labeled AOS were removed and counted by liquid scintillation, showing that aspirin acetylation of AOS coincided with the observed loss of enzyme activity (filled squares).

To identify the amino acid residue(s) involved, $^{14}C$-labeled protein was proteolytically cleaved, and the peptides were subjected to HPLC fractionation, sequencing, and scintillation counting. Three labeled peptide fragments were identified (Fig. 6). Peptide fragment #1, with the sequence (S$^*$)VVYESLRI-EPPVPPQYGK, was labeled in cpm as follows (bkg, background): (2000*)/100/75/65/50/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg (Fig. 6). Only the first of the two serine residues in this peptide was labeled, which represented Ser$^{332}$ in guayule AOS. Protein sequence comparisons with AOS from other species revealed that this serine was conserved among other plants and corresponded to Ser$^{395}$ in flaxseed AOS (34), Ser$^{375}$ in Arabidopsis AOS (35), and to Ser$^{347}$ in pepper hydroperoxide lyase (HPL) (36) (Fig. 7). The second labeled peptide fragment, #2, with the sequence SNFTIE(S$^*$)HDATFEVKK, was labeled in cpm to a lesser degree as follows: 55/75/40/60/50/(750*)/40/50/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg/bkg (Fig. 6). Only the second of the two serine residues in this peptide fragment was labeled, which represented Ser$^{359}$ in guayule AOS, Ser$^{422}$ in flaxseed AOS, Ser$^{402}$ in Arabidopsis AOS, and Ser$^{373}$ in pepper HPL (Fig. 7). The third labeled peptide fragment, #3, with the sequence YVWW(S$^*$)NG PEETE SPTVEN, was also weakly labeled in cpm, as follows:
1- or 2-h incubation with 14C-aspirin. Autoradiography. The 53-kDa AOS band was specifically labeled after a
substrates. Other P450s in this group include thromboxane
oxidases are noted for not accepting electrons from a protein-
acylate reducing partner. This gives them uncommonly high
turnover rates compared with classical P450s. Recombinant
AOS from guayule exhibits these same high turnover rates,
with $k_{\text{cat}}$ values in excess of 1000 s$^{-1}$ for each of the three
substrates tested, which is consistent with this class of cyto-
chrome P450s. It is surprising, however, that AOS shows
higher affinity for 15S-HPETO than it does for 13S-HPODE or
13S-HPOTE. These latter two substrates are normal plant
metabolites, whereas 15S-HPETO, being the predominant lipid
derivative in animals, is rarely encountered in plants (19, 10).
However, these data appear to agree with early results that
show that flaxseed AOS could metabolize a wide variety of lipid
hydroperoxides, including 15S-HPETO (39).

Our results show that NSAIDs act as competitive inhibitors
of AOS. This supports earlier findings (23, 24) that AOS is the
possible target of these inhibitors in blocking oxylipin synthe-
sis. It is noteworthy that HPL from sunflower has also been
reported to be inhibited 35% by SA (40). This is because it too,
is another form of a nonclassical P450 that happens to share
40% homology with AOS (36). Thus, AOS from plants and its
closely related enzyme, HPL, appear to possess features that
result in inhibition by salicylates. One exception to this obser-
vation may be a form of AOS from coral that did not appear to
be inhibited by indomethacin (41).

NSAIDs are not generally viewed as inhibitors of cytochrome
P450s (42). However, nitric oxide synthase, a heme thiolate
enzyme related to cytochrome P450, is reported to be inhibited
by aspirin and related NSAIDs (7). Indomethacin can also
inhibit at least one classical P450 P450 monoxygenase that is in-
volved in arachidonic acid metabolism (43, 44). Thus NSAIDs
such as aspirin, which are known to disrupt the arachidonic
cascade, may do so by affecting enzymes other than PGHS (43).

In animals, salicylates block autacoid formation by inhibiting
the cyclooxygenase activity of PGHS and do so by acetylat-
ing a single serine residue that resides near the C terminus of
the protein. The acetyl group blocks entry of the substrate in
the channel leading to the active site, causing irreversible
inhibition of the enzyme. In plants, although salicylates are
also known to block autacoid formation, it is not known if AOS
is inhibited by a similar mechanism. Plants do not possess
cyclooxygenase, and AOS has no sequence similarity to it.

This is the first known example of a cytochrome P450 to be
acyetylated and irreversibly inhibited by aspirin. Although as-
pirin at high quantities is known to acetylate other proteins
such as hemoglobin, the fact that low concentrations of aspirin
used here led to the irreversible inactivation that coincided
with its rate of labeling in vitro argues that acetylation may be
the reason for its inactivation. Moreover, the quantities of
radiolabeled aspirin used in our studies were identical to those
used in the original experiments demonstrating aspirin acety-
lization of animal PGHS (30). Except for a few isolated examples,
classical P450s do not become inhibited by salicylates and are
not expected to be acetylated by aspirin. Thus, we do not expect
aspirin to acetylate most other classical type P450s.

The best-studied target of aspirin is PGHS. Salicylates are
able to compete for the active site of PGHS despite having a
molecular structure that is completely different from the nor-
mal lipid substrate of the enzyme (1). Inactivation is caused by
the acetylation of a serine residue positioned some distance
from the active site in the substrate channel. This acetate
further blocks the entry of lipid substrates and causes the
irreversible inhibition of the enzyme (45–47). Because AOS
responds in a similar manner, it hints that aspirin inhibition
might be comparable with PGHS. The kinetics of aspirin inhi-

![Figure 5. Acetylation of AOS by aspirin. Labeling was performed as
described under "Experimental Procedures." Thirty µg of purified AOS
was incubated with 1 m M 14C-aspirin for 0-h (lane 1), 1-h (lane 2), and
2-h (lane 3) incubations at 37 °C and subjected to SDS-PAGE and
autoradiography. The 53-kDa AOS band was specifically labeled after a
1- or 2-h incubation with 14C-aspirin.](https://example.com/figure5)

![Figure 6. HPLC analysis of proteolysis products of 14C-aspirin-
labeled AOS. Three radioactively labeled peptide fragments were iden-
tified, as indicated by arrows on the HPLC elution profile. These were
collected and subjected to amino acid analysis and scintillation count-
ing. The sequence of each fragment is shown, and the most heavily
labeled residues from each fragment are indicated in parentheses.](https://example.com/figure6)
FIG. 7. Alignment of amino acids for AOS and HPL. Sequences are for AOS from flaxseed (1lu-aos), guayule (2pa-aos), and Arabidopsis (3at-aos), and for HPL from red pepper (4ca-hpl). Peptide fragments #1, #2, and #3 of guayule AOS are indicated (open bars) beneath the sequence. The 14C-acetylated serine residues for each of the labeled peptides are indicated (arrows) along with their radioactivity in cpm. The position of the K helix common to all P450s is denoted (shaded bar) as is the CAX heme binding site. Accession numbers: P48417 (1lu-aos), A56377 (2pa-aos), 1890152 (3at-aos), 1273240 (4ca-aos).

FIG. 8. Comparison of amino acid sequences in the K helix region of assorted cytochrome P450s. The labeled serine residue (*) of guayule AOS is conserved in AOS and HPL from other species, but serines are not found in other classical cytochrome P450s where these residues are typically Ala, Met, or Gln at this position. The highly conserved Glu and Arg residues (arrows) are found in all cytochrome P450s, including AOS and HPL.
bition on AOS are similar to PGHS, and the acetylated residues occur on serines found near the C terminus. If similar mechanisms are involved, one would expect that one or more of these acetylated serines would reside in the substrate channel of AOS. Unfortunately, crystal structures of AOS and other eukaryotic P450s are not currently available to verify this. However, three-dimensional structures for prokaryotic P450s (48, 49) were used in comparing sequence alignments between conventional P450s and for residues Ser332, Ser359, and Ser411 of guayule AOS and corresponding positions of AOS and HPL from other plant species (Fig. 8). The most heavily labeled residue, at Ser332, resides in the conserved K helix (50), or the so-called domain B (51), found in all P450s (Fig. 8). This residue lies 4 and 7 residues downstream of the highly conserved Glu and Arg residues, which are common to all P450s. These Glu and Arg residues are required for H bonding and normal enzyme function. Thus, it is conceivable that acetylation of this serine in AOS could disturb normal H bonding and possibly inactivate the enzyme. In BM3 (49), this residue is occupied by Met316, so it would not be affected by acetylation. However, serines occupy this position in AOS and HPL from other plant forms (Fig. 8), which are all thought to be inhibited by salicylates.

The two other serines we observed in guayule AOS at Ser359 and Ser411, which were labeled to a lesser extent, correspond to residues Gly342 and Ile385 of BM3. In BM3 these positions are found on the surface of the protein and are, thus, not suspected of affecting enzyme activity in such classical P450s. However, for other plant forms of AOS and HPL, these residues are conserved. Thus, we conclude that if these other residues contribute to enzyme inactivation. Because NSAIDs appear to act as competitive inhibitors, one would expect that these inhibitors should affect the substrate binding site of AOS. Without a three-dimensional structure, it is difficult to predict if this occurs or to be certain whether aspirin acetylation is responsible for the observed inhibition of AOS.

Salicylates occur naturally in plants and are known to regulate several important cellular processes in them (52–54). Salicylates are not produced in animals, but animals are greatly affected by them, largely through their effect on PGHS and autacoid synthesis. It is noteworthy that oxylipin synthesis in plants and animals follow parallel pathways, despite the involvement of different enzymes and mechanisms. It is interesting that salicylates act by blocking the same step of the pathway, yet do this by inhibiting two structurally different enzymes. In animals it is the non-P450 cyclooxygenase activity of PGHS (1), whereas in plants it is the peroxidase activity of AOS, a cytochrome P450. It appears that the control of autacoid biosynthesis across a wide spectrum of organisms is affected by salicylates at the same point in the pathway.

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