The mechanism of \( \omega \)-6 polyunsaturated fatty acid oxidation by wild-type cyclooxygenase 2 and the Y334F variant, lacking a conserved hydrogen bond to the catalytic tyrosyl radical/tyrosine, was examined for the first time under physiologically relevant conditions. The enzymes show apparent bimolecular rate constants and deuterium kinetic isotope effects that increase in proportion to co-substrate concentrations before converging to limiting values. The trends exclude multiple dioxygenase mechanisms as well as the proposal that initial hydrogen atom abstraction from the fatty acid is the first irreversible step in catalysis. Temperature dependent kinetic studies reinforce the novel finding that hydrogen transfer from the reduced catalytic tyrosine to a terminal peroxyl radical is the first irreversible step that controls regio- and stereospecific product formation.

Cyclooxygenases 1 and 2 (COX-1 and COX-2) are tyrosyl radical-utilizing hemoproteins with dioxygenase and peroxidase activities (1–3). Also known as prostaglandin H synthases, these structurally homologous enzymes are expressed by distinct genes, resulting in differences in cellular localization and regulation. COX-1 and COX-2 use O\(_2\) to oxidize arachidonic acid (AA) as well as linoleic acid (LA), its dietary precursor, by Equations 1 and 2. COX-1 has a smaller active site, resulting in greater specificity for AA over LA than seen in the larger, more promiscuous COX-2 (4).

Each dioxygenase reaction starts with initial hydrogen abstraction (from AA or LA) by a catalytic tyrosyl radical. Antarafacial O\(_2\) trapping (of AA’ or LA’) leads to a peroxyl radical that reoxidizes the catalytic tyrosine in a second hydrogen transfer step, thus propagating catalysis. The prostaglandin G\(_2\) (PGG\(_2\)) and acyclic hydroperoxide compounds (HPETEs and HPODEs) formed are processed by the enzyme’s peroxidase activity, which consumes two reducing equivalents to generate prostaglandin H\(_2\) (PGH\(_2\)) and acyclic hydroxylated compounds (hydroxyicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs)) together with H\(_2\)O. In vitro studies commonly use phenol as the reductant. Earlier work demonstrated simple, saturating behavior when phenol reacts with COX-2 (5) but not COX-1 where acceleration and inhibition of dioxygenase catalysis are observed upon raising the phenol concentration (6).

AA and LA bind to COX-2 in similar L-shaped conformations (Fig. 1). The reactive bisallylic C–H bond is thereby positioned close to the catalytic tyrosyl radical (Tyr-371) (7, 8). Tyr-371’ forms rapidly upon exposure of the enzyme containing Fe(III)(protoporphyrin IX (Por)) to hydroperoxide compounds present at trace levels in AA and LA preparations (9). A Fe(IV)=O(Por’') intermediate, typical in heme peroxidases, has been implicated as the oxidant (10). This species resides approximately 5 Å away from Tyr-371, which hydrogen bonds to Tyr-334 (11, 12). By analogy, Mn(III)(Por)-reconstituted COX-2 likely reacts with hydroperoxide compounds via a Mn(V)=O(Por) intermediate to produce Tyr-371’ (1).

Prior to findings outlined here, small substrate kinetic isotope effects (KIEs) were reported to characterize dioxygenase catalysis by COX-1 and COX-2 (13–15). Single turnover experiments with COX-1 indicated a deuterium KIE of ~2 upon AA consumption that was indistinguishable from the deuterium KIE upon the steady-state bimolecular rate constant, \( Dk_{cat}/K_m(AA) \). This KIE is determined by steps beginning with AA association proceeding up to and including the first irreversible step. The results above, suggesting that initial hydrogen abstraction is the first irreversible step in COX-1, have been extended to COX-2 because of a similar \( Dk_{cat}/K_m(AA) \) of ~2 extracted from state-state rate constants (4, 13, 15). This interpretation of the KIE makes it a challenge to explain the region- and stereospecific reactivity of O\(_2\) during dioxygenase catalysis, leading to the hypothesis that distortion of the substrate radical causes accumulation of unpaired spin at a position accessible to O\(_2\) via a channel in the protein (16). Since the works cited above, COX-1 and COX-2 have been reported to exhibit inverse deuterium KIEs and anomalous activation energies associated with \( Dk_{cat}/K_m(AA) \) as well as \( Dk_{cat} \). The latter has been proposed to reflect an inverse temperature dependence of the deuterium KIEs upon enzyme turnover at saturating concentrations of O\(_2\) (15). Such behavior might also arise from kinetic complexity due to competing
steps with different temperature dependences instead of the reportedly lower thermal activation barrier to deuterium transfer than protium transfer (17).

In the present work, new data collected under physiologic conditions argue that a second hydrogen transfer, downstream of AA’ or LA’ formation and O₂ trapping of the substrate radical, is the first irreversible step. In this reaction, the terminal peroxyl radical is reduced to the hydroperoxide product, and Tyr-371 is reoxidized to Tyr-371’. In the proposed mechanism, kinetic factors dictate regio- and stereospecific product formation during dioxygenase catalysis, a finding with implications for designing mechanism-based COX-2-specific inhibitors (18).

**Experimental Procedures**

**General Methods**—Chemicals were procured in the highest purity available and used as received unless noted. Sodium phosphate, sodium pyrophosphate, sodium chloride, sucrose, polyethylene glycol, and H₂O₂ were obtained from Fischer. Mn(III)(Por)Cl was obtained from Frontier Scientific. Hematin, soybean lipoxigenase, horseradish peroxidase, phenylmethylsulfonyl fluoride (PMSF), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, trimethylphosphite, n-alkanesulfonyl fluoride (PMSF), 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, Tween 20, allowed isolation of imidazole-free solution of COX-2 apo-proteins lacking the Fe(III)(Por). Alternatively, apoproteins were purified by dialysis for >12 h against the pH 8.0 buffer above.

**Protein Preparation and Characterization**—N-terminal His₆-tagged proteins were expressed recombinantly in baculovirus-transfected S9 insect cells following published protocols (19). Wild-type (WT), Y334F, and Y371F constructs (20) were sequenced and deposited at the Baylor College of Medicine Baculovirus/Monoclonal Antibody facility where proteins were expressed following amplification, titration, and Western blotting characterization (5).

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The following procedure was used to purify COX-2 proteins with all manipulations conducted at 4 °C unless noted. Cell pellets were suspended (1 g/4 ml) in a pH 7.4 buffer consisting of 25 mM NaH₂PO₄, 100 mM NaCl, 20 mM imidazole, and 1 mM PMSF prior to lysis. Next, sonication was performed using 50% amplitude and cycles of 10 s on and 60 s off. The lysate was isolated and centrifuged for 1 h at 43,000 × g. The supernatant was discarded, and the membrane fraction resuspended in the above lysis buffer supplemented with 0.1% (v/v) protease inhibitor mixture (Sigma) and Tween 20. The suspension was homogenized (Dounce), stirred on ice for 1.5 h, and then centrifuged for 1 h at 36,000 × g. The cell pellicl was discarded, and the supernatant was incubated with nickel-nitrilotriacetic acid resin pretreated with the lysis buffer for 90 min before loading onto a nickel-nitrilotriacetic acid affinity column (2-cm diameter, 10-ml bed volume). Chromatography utilized a pH 7.4 buffer containing 25 mM NaH₂PO₄, 20 mM imidazole, 0.1% (w/v) Tween 20, and 100 mM NaCl at a flow rate of approximately 0.65 ml/min. The ionic strength (μ) was raised using 300 mM NaCl prior to eluting the protein with 25 mM NaH₂PO₄, 100 mM NaCl, 0.1% (w/v) Tween 20, and 200 mM imidazole. Aliquots were removed and assayed for peroxidase activity. This procedure involved adding hematin, H₂O₂, and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt to samples followed by colorimetric analysis of COX-2 peroxidase activity. Fractions with peroxidase activity were pooled, loaded onto a PD-10 desalting column (Bio-Rad), and eluted with 100 mM NaH₂PO₄ at pH 7.0 or 16 mM Na₂H₂P₂O₇ at pH 8.0 in the presence of 10% (v/v) glycerol and 0.1% (w/v) Tween 20, allowed isolation of imidazole-free solutions of COX-2 apoproteins lacking the Fe(III)(Por). Alternatively, apoproteins were purified by dialysis for >12 h against the pH 8.0 buffer above.
The Dioxygenase Mechanism of Human COX-2

trated to ≤5 ml using an Amicon stirred cell equipped with a 30-kDa ultrafiltration membrane (Millipore), apportioned into 50–100-μl aliquots, and stored at −80 or −30 °C inside the freezer of the N₂-filled glovebox described above.

Protein samples were characterized by amino acid analysis (Texas A&M), polyacrylamide gel electrophoresis, and electronic absorption using a diode array spectrophotometer (HP8453a). The extinction coefficient for apo COX-2 (ε_{375 nm} = 116,000 M⁻¹ cm⁻¹) (21) was used to compute extinction coefficients associated with the Soret bands of Fe(III)(Por)- and Mn(III)(Por)-bound COX-2 proteins, which ranged from ε_{407 nm} = 129,000 M⁻¹ cm⁻¹ to ε_{375 nm} = 66,000 M⁻¹ cm⁻¹ and ε_{470 nm} = 42,000 M⁻¹ cm⁻¹, respectively.

Assessing Active Enzyme Concentration—The concentration of active COX-2 was analyzed using extinction coefficients calculated as outlined above and a standard assay conducted at 30.0 ± 0.2 °C. Standard assay buffers contained kinetically saturating concentrations (> 6 Kₘ) of the following dioxygenase and peroxidase substrates: [AA] or [LA] = 50–100 μM, [O₂] = 237 μM (from air), and [phenol] = >1.5 mM. Hematin or Mn(III)(Por) was added (1 μM) to prevent significant dissociation of the prosthetic group from the holoprotein. Initial rates were measured with an O₂ electrode (YSI model 5300A) and compared with predetermined kcat values. As expected, Y371F COX-2 showed no activity due to the absence of Tyr-371, whereas WT and Y334F COX-2 showed comparable kcat values (22).

Ascertaining the concentration of active enzyme is critical to the investigation of rate constants and deuterium KIEs at variable temperatures. In such experiments, changes in protein structure might result in loss of activity. Therefore, enzyme concentrations were checked by preincubating aliquots of stock solution at reaction temperatures between 5.0 and 50.0 °C for 3 min before performing standard assays. Under these conditions, no decrease in enzyme activity was detectable, and no correction for enzyme concentration was applied.

Steady-state Kinetics—Initial rates were measured at 30.0 ± 0.2 °C unless noted using the O₂ electrode (above). All experiments were conducted at pH 8.0 in 0.016 M sodium pyrophosphate (μ = 0.1 M) using known concentrations of AA or LA and O₂ along with optimized concentrations of phenol, hydroperoxide initiator, and 1 μM hematin or Mn(III)(Por)Cl.

Kinetic measurements were initiated by injecting 1–10 μl of enzyme stock solution into 1.0–1.5 ml of stirring reaction solution contained inside a chamber with no headspace. Standard assays were used to check for changes in enzyme concentration over the course of experiments. Diminution in dioxygenase activity is clearly detected as COX-2 undergoes turnover. However, initial rates extracted from the most linear portion of the reaction progress curve after allowing ~5 s for mixing varied in proportion to the enzyme concentration. This behavior is consistent with no significant loss of enzyme activity on the experimental time scale. In some cases, correction to the O₂ uptake rate for background drift of the YSI electrode was required. In no instance was this correction >30% of the measured rate.

Kinetic parameters were obtained by fitting data to the hyperbolic Michaelis-Menten equation: V/[E] = kcat[S]/(Kₘ + [S]) using Kaleidagraph 4.0 (Synergy Software). The concentration of fatty acid or O₂, [S], was varied, whereas the co-substrate concentration was held fixed at kinetic saturation; i.e. > 6 Kₘ. The apparent maximal rate (Vₘₐₓ) divided by the active enzyme concentration, [E], plotted versus [S] gave a hyperbolic trend that was fitted to obtain the unimolecular rate constant kcat and bimolecular rate constants kcat/Kₘ(LA) or kcat/Kₘ(AA) and kcat/Kₘ(O₂).

Studies of temperature-dependent kinetics at high and low [O₂] were performed using protiated and deuterated substrates. The resulting substrate deuterium KIEs upon kcat and kcat/Kₘ(O₂) were determined over a 5.0 or 10.0 to 50.0 °C range. Data were analyzed by linear regression of natural log versus inverse temperature plots assuming the phenomenological Arrhenius expression: kcat or kcat/Kₘ(O₂) = A exp(−E_a/RT).

Viscosity Effects—Experiments were carried out to probe for diffusion-limited contributions to kcat/Kₘ(O₂) in accord with the Stokes-Einstein relation (23). Initial rates were examined at varying [O₂] while [AA] or [LA] was held constant at 50–100 μM (> 6 Kₘ) in the presence of sucrose or polyethylene glycol, which served as micro- and macroviscosogens, respectively. An Ostwald viscometer allowed the relative viscosity, η/η₀ relative to water, to be measured and correlated to kcat/Kₘ(O₂) as a means of probing for diffusion-controlled steps (24, 25). The effect of added micro- or macroviscosogen was examined up to η/η₀ = 4 to test whether O₂-trapping of AA or LA and hydroperoxide product release steps contribute to kcat/Kₘ(O₂).

Product Analysis—Products of AA and LA oxidation were quantified in relation to the O₂ consumed to confirm the reaction stoichiometries of Equations 1 and 2. Experiments were carried out by adding enzymes to 3–10-ml reaction solutions. After monitoring O₂ uptake, 1 ml of acetic acid was added to quench the reaction. Acidification also neutralized the fatty acid oxidation products, allowing for extraction into CH₂Cl₂. Although COX-2 possesses peroxidase activity, 5% (v/v) trimethyl phosphite was added to ensure reduction of hydroperoxide products; i.e. PGG₂ was converted to PGH₂, HPETEs were converted to HETEs, and HPODEs were converted to HODEs (26). Solutions of these compounds were evaporated to a residue under a stream of N₂ and then redissolved in a known volume of methanol. Samples were analyzed by ultra-high-performance liquid chromatography (UPLC) on a Waters Acquity/Xevo-G2 system equipped with a reverse phase C₁₈ column (HSS T3 1.8 μm, 2.1 × 100 mm) and quadrupole time-of-flight mass spectrometer operating in negative ion mode. The mobile phase (60% acetonitrile and 40% water), flowing at a rate of 0.5 ml/min, allowed separation of products, which were identified based on comparisons with authentic samples.

Competitive Deuterium KIEs—Apparent deuterium KIE upon LA oxidation, ap ΔD kcat/Kₘ(LA), was measured competitively in 100 μM solutions of perprotiated (h₃₃-LA) and perdeuterated (d₃₃-LA) fatty acid combined in a 1:3 ratio. Experiments were performed with WT and Y334F COX-2 over a range of O₂ concentrations by analyzing the ratio of deuterated to protiated products at low conversion (~10%) relative to 100% conversion. The latter measurements used excess COX-2 or soybean lipoxgenase (26). Following the protocol above, UPLC-MS exposed two baseline resolved signals corresponding to the
monodeprotonated isomers of $h_{31}$-HODE and $d_{31}$-HODE. This ratio was corroborated by analysis of electronic absorption at 235 nm, the wavelength of maximum absorption associated with the conjugated diene in the product.

**Solvent Isotope Exchange**—Experiments were undertaken to test whether the reactive hydrogen of the substrate undergoes exchange with the solvent in the presence and absence of Tyr-371*-containing COX-2. WT, Y334F, and Y371F proteins were incubated anaerobically in 3–10 ml of N$_2$-saturated pH 8 buffered H$_2$O containing 50 $\mu$M $d_{31}$-LA. HPODEs in this substrate were present at trace levels sufficient to ensure full enzyme activation. 2 mM phenol was also added to protect enzymes from overoxidation by HPODEs. Samples were incubated at 4°C under N$_2$ for 12 h before reisolation of unreacted from overoxidation by HPODEs. Samples were incubated at 4°C under N$_2$ for 12 h before reisolation of unreacted.

**Results**

Recently, Danish et al. (5) demonstrated that the dioxygenase activity of COX-2 could be analyzed independently of the peroxidase activity of the enzyme at sufficiently high concentrations of phenol. Such investigations are not possible with COX-1 where rate acceleration is followed by inhibition upon adding phenol (6). However, rates of dioxygenase catalysis were found to saturate hyperbolically upon increasing the phenol concentration to 1.0–3.0 mM with Fe(III)(Por)-containing WT COX-2 and Y334F COX-2. With these enzymes, trace hydroperoxide impurities present in AA or LA were sufficient to observe optimal rates of turnover. The lower peroxidase activity of Mn(III)(Por)-WT COX-2 eliminates the requirement of added phenol. Even in the presence of 3–10 $\mu$M HPODE or HPETE needed to optimize initial rates, no phenol dependence was detected. Furthermore, the inhibition of dioxygenase activity by phenol was undetected in all forms of COX-2 examined in this study.

**LA Oxidation Kinetics**—Steady-state rate constants for LA oxidation were determined for WT COX-2 and the Y334F variant, which lacks a conserved hydrogen bond to Tyr-371* or Tyr-371 (7, 8, 11, 12). In Figs. 2 and 3, the apparent rate at kinetically saturating co-substrate concentration ($\geq$6 $K_m$) is given as $V/\text{[E]}$ and plotted versus varied substrate to determine $k_{cat}/K_m$ and $k_{cat}$ as outlined under “Experimental Procedures.”

Rate constants summarized in Table 1 show that WT and Y334F COX-2 exhibit similar $k_{cat}$, $k_{cat}/K_m$(AA), and $k_{cat}/K_m$(LA). Comparisons of AA with LA require correction by a factor of 2 for the difference in O$_2$ equivalents consumed in Equations 1 and 2. Quantification of dioxygenase products in tandem with measurements of O$_2$ uptake confirmed the stoichiometry for formation of PGH$_3$ as the major product of AA oxidation and HODEs as the products of LA oxidation. The $k_{cat}/K_m$(O$_2$) was, however, an order of magnitude greater for AA than LA due to a reduction in $K_m$(O$_2$).

**Deuterium KIEs on LA Oxidation**—The apparent rate constants for consumption of $h_{31}$-LA versus $d_{31}$-LA afford deuterium KIEs that increase with [O$_2$] until a limiting $k_{cat}/K_m$(LA) is attained. Most measurements were made competitively by mixing the substrate isotopologues and analyzing the $h_{31}$-HODE to $d_{31}$-HODE product ratios after reduction of $h_{31}$- and $d_{31}$-HPODEs following a published procedure (26). Apparent competitive deuterium KIEs upon LA oxidation, $^{ap}k_{cat}/K_m$(LA), were found to increase from subsaturating to saturating [O$_2$] in Fig. 4. This behavior is in line with the apparent deuterium KIEs determined non-competitively by measuring O$_2$ uptake rates with $h_{31}$-LA or $d_{31}$-LA (see Table 1).

Remarkably, the $^{ad}k_{cat}/K_m$(LA) values for WT and Y334F COX-2 are equivalent to the deuterium KIEs upon bimolecular rate constants for O$_2$ uptake, $^{ap}k_{cat}/K_m$(O$_2$), determined under non-competitive conditions. This unusual deuterium KIE upon O$_2$ consumption arises from retention of the hydrogen abstracted from LA at Tyr-371; solvent exchange with this residue is slow on the time scale of enzyme turnover (see below).

**Equations 1 and 2.**

1. $V/\text{[E]} = \frac{k_{cat}}{K_m}$
2. $k_{cat} = \frac{V}{\text{[S]}}$

**FIGURE 2. Initial rates of O$_2$ consumption used to determine $^{ap}k_{cat}/K_m$(O$_2$) for dioxygenase catalysis by WT (circles) and Y334F (squares) COX-2.** The KIE was extracted from data independently fitted to Michaelis-Menten expressions. The [O$_2$] was varied, and $[h_{31}$-LA] or $[d_{31}$-LA] was maintained at 100 $\mu$M (i.e., $>6K_m$; see Table 2) in sodium pyrophosphate (0.016 M) at 30°C, pH 8.0, and $\mu = 0.1$ M as detailed under “Experimental Procedures.”
Limiting rate constants are quoted with rate constants for oxidations of AA and LA at 30 °C
M) at 30 °C, pH 8.0, and
M as detailed under “Experimental Procedures.”

All solutions initially contained h31-LA and d31-LA mixed in a ratio of 1:3, respectively, at a concentration of 100 μM. The h31-HODE/d31-HODE ratio at <10% reaction conversion was taken relative to the h31-HODE/d31-HODE ratio at 100% conversion to determine the ap Dcat/Km(O2). These apparent KIEs were fitted to the Michaelis-Menten equation to obtain the limiting competitive Dcat/Km(LA) which is compared with the corresponding Dcat/Km(O2) determined in Fig. 2. Error bars are 1 S.D.

The observed equivalence of Dcat/Km(LA) to Dcat/Km(O2) is consistent with a common irreversible hydrogen transfer in WT and Y334F COX-2 after LA and O2 enter the catalytic cycle.

Table 2 consists of data obtained with two forms of COX-2 containing a Fe(III)(Por) or Mn(III)(Por) prosthetic group. The WT COX-2 exhibits a Dcat/Km of ~21, which is 2–3 times larger than Dcat/Km(LA) and Dcat/Km(O2). These results, together with reported temperature dependences of Dcat and Dcat/Km, expose a change in the irreversible hydrogen transfer step as the [O2] is raised (5). In contrast, Y334F COX-2 exhibits a Dcat of ~30 that is indistinguishable from Dcat/Km(LA) and Dcat/Km(O2) within the error limits.

The large [O2]- and [LA]-independent deuterium KIEs implicate a common irreversible and rate-controlling step for Y334F COX-2 turnover at all substrate concentrations. Temperature studies of Dcat and Dcat/Km(O2) described below also support a common irreversible step. In addition, competitive oxygen-18 KIEs upon kcat/Km(O2), reflecting steps that begin with O2 encounter and lead up to and include the first irreversible step, are indistinguishable for WT and Y334F COX-2 catalysis with LA (1.0133–1.0156) and AA (1.0194–1.0205). These results, which correlate the magnitude of 18O KIE to the extent of peroxyl radical reduction in the transition state (27, 28), suggest common irreversible steps in WT and Y334F COX-2 at physiological [O2] (see below).

Solvent Isotope Exchange—Under anaerobic conditions, hydroperoxide-activated WT and Y334F COX-2 containing Tyr-371’ catalyze exchange of a single proton from H2O into

TABLE 1
Rate constants for oxidations of AA and LA at 30 °C

|            | COX-2 |      |      |
|------------|-------|------|------|
|            | WT    | Y334F|      |
|            |       |      |      |
| h31-LA     |       |      |      |
| kcat/km    | 9.4 (0.8) | 8.0 (0.8) |      |
| kcat/(O2)  | 1.6 (0.2) | 1.4 (0.2) |      |
| kcat/(O2)  | 0.050 (0.010) | 0.047 (0.008) |      |
| d31-LA     |       |      |      |
| kcat/km    | 0.44 (0.03) | 0.27 (0.02) |      |
| kcat/(O2)  | 0.15 (0.02) | 0.050 (0.006) |      |
| kcat/(O2)  | 0.0075 (0.0007) | 0.0016 (0.0002) |      |
| h31-AA     |       |      |      |
| kcat/km    | 16.7 (2.0) | 8.5 (1.0) |      |
| kcat/(O2)  | 5.9 (0.5) | 3.0 (0.3) |      |
| kcat/(O2)  | 1.1 (0.4) | 0.7 (0.2) |      |

* kcat/Km(39-AA) is close to the detection limit; thus, kcat/Km(32P- AA) should be viewed as an upper limit.
Deuterium KIEs upon LA oxidation at 30 °C

| COX-2 | WT | WT(Mn(III)) | Y334F |
|-------|----|-------------|-------|
| $d_{31}$-LA | 21.4 (2.3) | 20.0 (3.0) | 29.6 (3.6) |
| $d_{31}$-Km | 10.6 (1.9) | 8.0 (1.0) | 28.0 (3.4) |
| $d_{31}$-Km(LA) | 8.7 (0.8) | 7.5 (1.0) | 25.0 (3.2) |
| $d_{31}$-Km(O$_2$) | 6.7 (1.4) | 11.0 (2.6) | 29.4 (4.2) |

* Determined competitively by UPLC-MS analysis of the products at <10% of the expected O$_2$ consumed as described under “Experimental Procedures.”

Deuterium KIEs upon AA Oxidation—In view of the large deuterium KIEs associated with LA-dependent O$_2$ consumption, $D_{cat}$ was examined for AA using unlabeled (h$_{33}$-AA) and selectively labeled (13,13-$d_{31}$-AA) substrates. No artificial burst in O$_2$ uptake was detectable with 13,13-$d_{31}$-AA, and initial rates were identical when this material was used as received and after preincubation with WT COX-2. These results indicate that any unlabeled material present in the 13,13-$d_{31}$-AA has no impact on the deuterium KIE.

$D_{cat}$, $K_m(O_2)$, and $K_m$ were determined from the data in Fig. 6. Three independent trials were performed by varying [O$_2$] and the resulting rates were analyzed as outlined under “Experimental Procedures.” Linear regression analysis at [O$_2$] < $1/2K_m(O_2)$ gave a $D_{cat}/K_m$ of 17.4 ± 3. Data obtained over a range of [O$_2$] and fitted to the Michaelis-Menten equation indicate a $D_{cat}/K_m$ of 18.6 ± 3.1 and a $D_{cat}$ of 3.1 ± 0. As discussed below, the large $D_{cat}/K_m$ is most readily attributed to irreversible hydrogen transfer from Tyr-371 to a peroxyl radical formed after O$_2$ trapping at the terminal carbon radical intermediate (29). Evidently, this rather large deuterium KIE escaped detection in earlier studies because saturating [O$_2$] was used in experiments (13–15, 29–32) rather than physiologic [O$_2$], which is approximately 50 μM (33).

Probing Viscosity Effects on $O_2$ Consumption—The kinetics of AA and LA oxidation analyzed at variable viscosities failed to provide evidence of diffusion-controlled contributions to $k_{cat}/K_m$. No change in the bimolecular rate constant for O$_2$ uptake was discernible with AA or LA in the presence of micro- and macroviscosogens. The effect of relative viscosity as high as 4 was examined to exclude irreversible O$_2$ trapping and hydroperoxide product release steps (34). In the respective cases, $k_{cat}/K_m$ would be expected to decrease and increase with increasing η/η.$0$.

Temperature Dependence of Deuterium KIEs—The temperature dependences of $D_{cat}$ and $D_{cat}/K_m$ were measured for

Isotope exchange from H$_2$O into $d_{31}$-LA takes several hours in the presence of WT and Y334F COX-2 because the active site is not accessible to the solvent. No exchange occurs on the same time scale in the presence of O$_2$. Therefore, the results in Fig. 5 indicate that hydrogen transfer between Tyr-371 and LA is thermodynamically reversible but do not address the kinetic reversibility of this reaction in the presence of O$_2$. Deuterium KIE upon AA Oxidation—In view of the large deuterium KIEs associated with LA-dependent O$_2$ consumption, $D_{cat}/K_m(O_2)$ was examined for AA using unlabeled (h$_{33}$-AA) and selectively labeled (13,13-$d_{31}$-AA) substrates. No artificial burst in O$_2$ uptake was detectable with 13,13-$d_{31}$-AA, and initial rates were identical when this material was used as received and after preincubation with WT COX-2. These results indicate that any unlabeled material present in the 13,13-$d_{31}$-AA has no impact on the deuterium KIE.

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Temperature Dependence of Deuterium KIEs—The temperature...
AA and LA using WT and Y334F COX-2. Data published for WT COX-2 (5) are compared with those determined for the Y334F variant in Fig. 7. These two enzymes exhibit similar competitive $^{18}$O KIEs as described above with each fatty acid substrate, suggesting that the transition state is the same for the step that limits $k_{cat}/K_m(O_2)$ (28, 35). This result is consistent with a common step giving rise to $Dk_{cat}/K_m(O_2)$ when the hydrogen initially abstracted from the substrate is retained at Tyr-371 and followed by Tyr-371' formation via irreversible hydrogen transfer after $O_2$ enters the catalytic cycle.

Previous findings on LA oxidation by WT COX-2 indicated a change in the first irreversible step upon increasing $[O_2]$ from below $K_m(O_2)$ to $>6K_m(O_2)$ (5). The activation energy ($E_a$) associated with $k_{cat}/K_m(O_2)$ and $k_{cat}$ was determined for h$_{31}$-LA and d$_{31}$-LA reacting with Y334F COX-2. In contrast to WT COX-2, the variant shows indistinguishable deuterium KIEs upon $k_{cat}$, $k_{cat}/K_m(LA)$, and $k_{cat}/K_m(O_2)$ with each approaching 30 at 30°C. This indication of kinetically simple behavior where the rate constant for dioxygenase catalysis is controlled by a common irreversible step is also consistent with the negligible $\Delta E_a$ associated with $Dk_{cat} = 2.7 \pm 0.7$ kcal mol$^{-1}$ and $k_{cat}/K_m(O_2) = 2.2 \pm 0.6$ kcal mol$^{-1}$ derived from data at saturating and sub saturating $[O_2]$, respectively, where $E_a(d_{31}$-LA) = 9.4 ± 0.7 or 7.9 ± 0.3 and $E_a(h_{31}$-LA) = 6.7 ± 0.7 or 5.7 ± 0.5. At low $[O_2]$, where results reveal irreversible hydrogen transfer from Tyr-371 to an $O_2$-derived peroxyl radical, Y334F COX-2 exhibits a $\Delta E_a$ that is 2 times smaller than that reported for the WT enzyme where $\Delta E_a = 5.0 \pm 0.8$ kcal mol$^{-1}$ and $A_{31}/A_{15}$ is much more inverse (5). These results must be reconciled with the proposal of a common irreversible step in WT and Y334F COX-2 under conditions where the enzymes exhibit indistinguishable competitive $^{18}$O KIEs and $Dk_{cat}/K_m(O_2)$ equal to $Dk_{cat}/K_m(LA)$.

The availability of 13,13-$d_2$-AA allowed $Dk_{cat}$ and $Dk_{cat}/K_m(O_2)$ to be examined over a limited temperature range in Fig. 8. Experiments were conducted at [phenol] of ~2 mM where optimal dioxygenase activity was observed. The parallel lines in the Arrhenius plots indicate normal temperature dependences of $Dk_{cat}$ and $Dk_{cat}/K_m(O_2)$.

Analyzing rate constants associated with WT COX-2 oxidizing AA at subsaturating and saturating [AA], respectively, indicate that $E_a(13,13-d_2$-AA) = 15.2 ± 2.0 and 15.4 ± 0.5 kcal mol$^{-1}$ and $E_a(h_{31}$-AA) = 13.2 ± 1.4 and 13.9 ± 0.9 kcal mol$^{-1}$. Subtracting $E_a$ for labeled AA from $E_a$ for unlabeled AA results in a $\Delta E_a$ of 2.0 ± 2.4 at low $[O_2]$ and 1.5 ± 1.1 kcal mol$^{-1}$ at high [O$_2$]. These results contradict a recent report by Wu et al. (15) in which deuterium KIEs were found to vary inversely with temperature at pH 7.0, resulting in $E_a(h_{31}$-AA) > $E_a(13,13-d_2$-AA). Importantly, the same source of 13,13-$d_2$-AA used earlier (13, 15) was used in the present study. In the earlier study, no attention was devoted to optimizing the [phenol] required to observe maximal rates at different temperatures. This oversight might cause kinetic complexity that results in the aberrant $\Delta E_a$.

Data reported previously (5) and in this study are compiled in Table 3. Arrhenius analyses suggest a higher thermal barrier to the oxidation of AA than the oxidation of LA. As discussed below, these observations could reflect a greater energy barrier due to reorganization of AA in the active site of COX-2 as well as a mechanism that involves additional pre-equilibrium steps before the first irreversible hydrogen transfer step that forms PGG$_2$ and regenerates Tyr-371'.

**Discussion**

The similar distributions of products formed by COX-1 and COX-2 suggest that the enzymes react by common mechanisms. There are fundamental differences in their substrate specificity profiles, however, because of the larger active site in COX-2 (1). In this study, dioxygenase catalysis with LA to produce HPODEs was examined as a model for HPETE formation from AA, which likely results from the same initial steps as PGH$_2$.

**Analyses of Steady-state Kinetic Parameters**—In the works cited above (5, 6), COX-1 and COX-2 exhibit apparent second order rate constants that depend upon co-substrate concentrations in a hyperbolic manner. To the best of our knowledge, the
present studies are the first to focus upon how substrate deuterium KIEs in COX respond to changes in [O2]. Although COX-1 reacts with high specificity for AA over LA, COX-2 exhibits nearly identical kcat, kcat/Km(AA), and kcat/Km(LA) when a correction is applied for the O2 consumed. In contrast, kcat/Km(O2) is an order of magnitude greater for AA than LA. This difference is due to a diminution in kcat when a correction is applied for the O2 consumed. In contrast, kcat/Km(O2) is an order of magnitude greater for AA than LA. This difference is due to a diminution in kcat when a correction is applied for the O2 consumed.

Deuterium KIEs upon LA Oxidation—The apDkcat/Km(LA) and apDkcat/Km(O2) exhibit hyperbolic dependences upon co-substrate concentrations analogous to that seen for apparent rate constants (5, 6). Each increases to a limiting deuterium KIE upon raising the [O2]. Published derivations (36, 37) indicate that in (i) the apDkcat/Km(LA) should decrease upon raising [O2] and in (ii) the apDkcat/Km(O2) should be O2-independent.

Findings with WT and Y334F COX-2 expose an increase in apDkcat/Km(LA) upon raising the [O2] until a limiting Dkcat/Km(LA) equal to Dkcat/Km(O2) is reached. This behavior is explained by the random kinetic mechanism in Fig. 9 under the simplifying assumptions that reversible LA binding couples to reversible hydrogen transfer and that the reactions exhibit negligible equilibrium isotope effects. These assumptions allow use of the previously derived Equation 3 as a starting point (37). Considering the limit where [O2] approaches 0 results in Equation 4, taking the limit where [O2] approaches 0 gives Equation 5.

\[
\frac{k_{cat}}{K_M} \text{(d31-LA, D)} = 1 + \frac{H_{k_5}(1 + k_2[O2]/k_1)}{k_4 + k_2(1 + k_2[O2]/k_1)}
\]

A ternary complex is hypothesized to result from O2 binding to LA* in Fig. 9. This intermediate, consisting of the reduced catalytic Tyr-371 and LA-derived peroxyl radical, is expected to react by irreversible hydrogen transfer to form either 9-HPODE. In competition, the ternary complex could release O2 from the LA-derived peroxyl radical via k-2. Alternatively, O2 could dissociate from the peroxyl radial and partition into an active site pocket in COX-2, and reverse hydrogen transfer from Tyr-371 to LA* could release LA via k-4. When k-4 ≫ k-2, raising [O2] results in an increase in the apDkcat/Km(LA) to the intrinsic KIE defined by \( \frac{H_{k_5}}{D_{k_5} + \frac{H_{k_5}}{k_2}} \). This scenario reproduces the trends in Fig. 4 in accord with Equations 3–5.

The mechanism also allows for observations that Dkcat/Km(LA) is equivalent to Dkcat/Km(O2). The random kinetic mechanism in Fig. 9 predicts that the deuterium KIE approaches a minimum as the [O2] is lowered. At the lowest [O2], deviation from unity is expected when the equilibrium isotope effect upon initial hydrogen transfer is significant. A negligible effect is anticipated in COX-2 because of the similar vibrational

TABLE 3

Activation parameters for AA and LA oxidation by WT and Y334F COX-2

|                | E_a(H) | E_a(D) | A_h | A_d |
|----------------|--------|--------|-----|-----|
| WT COX-2 + AA  | 13.9(9.9) | 15.4(6.6) | 2.3(5.5) \( \times 10^{12} \) | 9.1(4.1) \( \times 10^{11} \) |
| kcat/Km(O2)_a  | 13.2(1.4) | 15.2(2.0) | 8.6(30) \( \times 10^{15} \) | 8.5(13) \( \times 10^{15} \) |
| WT COX-2 + LA* | 9.1(0.2) | 9.1(0.2) | 3.6(1.2) \( \times 10^{7} \) | 1.8(1.1) \( \times 10^{6} \) |
| kcat/Km(O2)_a  | 7.3(0.6) | 12.3(0.5) | 6.8(2.7) \( \times 10^{4} \) | 4.9(2.8) \( \times 10^{3} \) |
| Y334F COX-2 + LA| 6.7(0.7) | 9.4(0.7) | 4.6(1.5) \( \times 10^{5} \) | 1.8(0.8) \( \times 10^{6} \) |
| kcat/Km(O2)_a  | 5.7(0.5) | 7.9(0.3) | 5.6(2.4) \( \times 10^{6} \) | 8.1(1.2) \( \times 10^{6} \) |

\( ^a \) Units of kcal mol\(^{-1} \).
\( ^b \) The units are the same as those of the rate constants.
\( ^c \) Units of s\(^{-1} \).
\( ^d \) Units of M\(^{-1} \) s\(^{-1} \).
\( ^e \) From Ref. 5.
The Large Deuterium KIE on AA Oxidation—The present results reveal a large $Dk_{cat}/K_m(O_2)$ when oxidation of $h_{19}$-AA is compared with 13,13-$d_2$-AA. As with LA, this result requires the initially abstracted hydrogen to be retained at Tyr-371 during catalysis with AA to Tyr-371'. Evidence that initial hydrogen transfer from AA to Tyr-371 might be reversible comes from earlier work in which the AA' was detected at vanishingly low $[O_2]$ (12).

A large $Dk_{cat}$ of $\sim 21$ and smaller $Dk_{cat}/K_m(O_2)$ of $\sim 7$ are observed during LA oxidation by WT COX-2. This behavior has been attributed to a change in the irreversible hydrogen transfer step upon varying $[O_2]$. A similar change in the first irreversible step might also occur during AA oxidation where $Dk_{cat}$ is $\sim 3$ and $Dk_{cat}/K_m(O_2)$ is $\sim 18$. Alternatively, an ordered sequential mechanism where AA binding and formation of AA' occur before $O_2$ is consumed might account for the difference in deuterium KIEs. Assignment of the mechanism requires examining apparent competitive KIEs at varying $[O_2]$.

The results highlighted in this study pertain to physiologically relevant conditions for the oxidation of LA and AA by COX-2. Previously, $Dk_{cat}/K_m(AA)$ was estimated to be $\sim 2$ in non-competitive experiments at an $[O_2]$ of $\sim 250$ $\mu$M; however, the use of non-optimal [phenol] could cause kinetic complexity under these conditions (5). Inhibition of dioxygenase activity by peroxidase turnover could also obscure $Dk_{cat}/K_m(AA)$ so that it is smaller than the intrinsic $H_k^+H_k^-Dk_k$ in Fig. 9.

The $Dk_{cat}/K_m(O_2)$ for AA is proposed to reflect Tyr-371 oxidation by the terminal peroxy radical. It is unclear, however, if the deuterium KIE arises from pre-equilibrium hydrogen transfer (from AA to Tyr-371') coupled to reversible 5-exocyclization steps in Fig. 9. Although $Dk_{cat}/K_m(O_2)$ exposes irreversible hydrogen transfer from Tyr-371 to PGG$_{2o}^+$, the possibility that $O_2$ equivalents are consumed independently cannot be rigorously excluded. Such a reaction seems unlikely in view of the greater $E_a$ and larger $^{18}O$ KIE associated with dioxygenase catalysis. If two irreversible reactions consume $O_2$ during AA oxidation, the observed $^{18}O$ KIE would be an average reflecting the $^{16}O$ KIE upon $O_2$ trapping of AA' followed by irreversible 5-exocyclization and the $^{18}O$ KIE upon $O_2$ trapping at the 15-25 position followed by reduction of PGG$_2^+$. This averaged $^{18}O$ KIE should be smaller than that observed with LA where the evidence points to an $^{18}O$ KIE arising from reversible $O_2$ trapping of LA' followed by irreversible reduction of the terminal peroxy radical by Tyr-371. Based on earlier experiments and computations (27, 35), the size of the $^{18}O$ KIE should increase in response to polarization of the hydrogen transfer to a proton/deuterium (H'/$D'$)-coupled electron transfer-like transition state (38). Such reactivity could explain the moderate to large $Dk_{cat}/K_m(O_2)$ and competitive $^{18}O$ KIEs measured with AA and LA as well as the absence of viscosity effects upon $k_{cat}/K_m(O_2)$, which reveals thermally activated hydrogen transfer rather than diffusion control.

Temperature Studies of Dioxygenase Catalysis—The temperature-dependent rate constants for WT and Y334F COX-2 summarized in Table 3 contradict findings recently reported by Wu et al. (15) that $E_a(h_{19}$-AA) significantly exceeds $E_a(13,13-d_2$-AA). Such results conflict with expectations that in the normal thermodynamic range tunneling is more probable for protium than deuterium (39) and requires less reorganization to achieve the reactive configuration. These properties make $E_a$ smaller for the lighter isotope of hydrogen. Although there are instances where inversely temperature-dependent deuterium KIEs have been attributed to hydrogen tunneling, this generally requires a highly exergonic “inverted region” in which the reorganization energy is offset by very favorable Gibbs free energy ($\lambda \equiv -\Delta G^\circ$) (17). This situation is unlikely in COX-2 where...
The Dioxygenase Mechanism of Human COX-2

oxidation of Tyr-371 by a peroxyl radical is estimated to be thermoneutral, $\Delta G^0 \sim 0$, because of the similar O–H bond strengths in Tyr-371 and the hydroperoxide product (40).

It is unsurprising that hydrogen transfer from Tyr-371 to the terminal peroxyl radical derived from LA is associated with a smaller $E_a$ than the analogous reaction of AA. Following antarafacial $O_2$ trapping of LA* or AA*, rearrangement of the terminal peroxyl radical must occur to remove hydrogen from Tyr-371. The hydrogen transfer that limits $k_{cat}/K_m(O_2)$ is expected to require less thermal activation to reorganize the smaller 9R or 13S peroxyl radical derived from LA than the larger PGG$_2^\bullet$ derived from AA, although multiple explanations are possible.

The reduction of PGG$_2^\bullet$ might take place over a shorter distance than the 9R or 13S peroxyl radical, explaining the larger magnitude of the Arrhenius prefactor ($A$). Favorable pre-equilibria could also elevate $A$ to a value approaching $10^{16} \text{M}^{-1} \text{s}^{-1}$ in the case of AA (see Table 3). Although the temperature dependence of $k_{cat}$ also indicates a large $A$ for AA compared with LA, the value of $10^{11} - 10^{12} \text{ s}^{-1}$ falls within the upper limit defined by transition state theory where $A$ is associated with $k_B T/h$ value of $\sim 10^{13} \text{ s}^{-1}$ where $\kappa$ is a probability factor related to hydrogen tunneling, $k_B$ is the Boltzmann constant, $T$ is temperature, and $h$ is the Planck constant.

Catalysis by WT and Y334F COX-2—Oxidation of Tyr-371 by the oxidized prosthetic group is slower in Y334F COX-2 than in the WT enzyme (22); however, there are no major differences in the steady-state rate constants of the enzymes (see Table 1). This behavior is consistent with the observed deuterium and $^{18}$O KIEs but raises questions concerning how their magnitudes might depend upon polarization of the hydrogen transfer in transition states with differently sized AA- and LA-derived peroxyl radicals (27, 35).

Mutating Tyr-334 to Phe reduces complexity of the enzyme kinetics and unmasks a single irreversible step controlling LA oxidation. The evidence for this change is inflation of $^{D}k_{cat}$, $^{D}k_{cat}/K_m(LA)$, and $^{D}k_{cat}/K_m(O_2)$ to values approaching 30. Additional support comes from temperature studies that reveal similar $E_a$ for $k_{cat}$ and $k_{cat}/K_m(O_2)$. This behavior is consistent with irreversible hydrogen transfer from Tyr-371 to the LA-derived peroxyl radical.

In contrast to Y334F COX-2, the WT enzyme exhibits a $^{D}k_{cat}$ of $\sim 21$ along with $^{D}k_{cat}/K_m(LA)$ and $^{D}k_{cat}/K_m(O_2)$ values that are 3 times smaller. Complementary temperature studies indicate disparate $\Delta E_a$ associated with $^{D}k_{cat}$ and $^{D}k_{cat}/K_m(O_2)$, implicating a change in the irreversible hydrogen transfer step. At high $[O_2]$, the KIE reflects initial hydrogen transfer from LA to Tyr-371*, whereas at low $[O_2]$, initial hydrogen transfer becomes reversible, and the second hydrogen transfer where the peroxyl radical is reduced by Tyr-371 becomes irreversible. This behavior is different in Y334F COX-2 where $E_a$ and $\Delta E_a$ are invariant to the $[O_2]$.

Although the magnitude of $k_{cat}/K_m(O_2)$ is essentially the same for WT and Y334F COX-2 oxidizing LA, a 4-fold increase in $^{D}k_{cat}/K_m(O_2)$ is observed. This is possibly the result of expanding the hydrogen transfer distance upon removal of the conserved hydrogen bond to Tyr-371. The smaller $E_a$ observed for Y334F COX-2 relative to WT COX-2 could be associated with greater protein flexibility accommodating reorganization of enzyme-bound intermediates required for hydrogen transfer from Tyr-371 to the terminal peroxyl radical.

Conclusions—Conditions have been identified where dioxygenase and peroxidase catalysis can be examined independently in recombinant human COX-2. This feature allows for the analyses of apparent rate constants and deuterium KIEs at variable co-substrate concentrations to address the kinetic mechanism of the enzyme. Studies of dioxygenase catalysis by Y334F COX-2, which lacks a conserved hydrogen bond to the catalytic Tyr-371 and Tyr-371*, reveal kinetically uncomplicated behavior. Using the Y334F variant together with the simpler reacting LA substrate exposes a random sequential mechanism and hydrogen transfer from Tyr-371 to a terminal peroxyl radical in the first irreversible step.

Temperature-dependent deuterium KIEs at subsaturating $[O_2]$ revealed differences in the behaviors of WT and Y334F COX-2. In both enzymes, the deuterium KIE upon $k_{cat}/K_m(O_2)$ arises from retention of the hydrogen abstracted from the substrate at Tyr-371 in the absence of solvent isotope exchange. In the first irreversible step, the terminal peroxyl radical is proposed to accept the hydrogen retained at Tyr-371 via a polarized hydrogen transfer or $H^+/H^+$-coupled electron transfer-like transition state. The less temperature-dependent $^{D}k_{cat}/K_m(O_2)$ observed for Y334F COX-2 relative to the WT enzyme suggests that removal of the conserved hydrogen bond creates greater protein flexibility or lower reorganization energy within the active site.

In this study, the reactivity of WT COX-2 with AA was also examined at physiologically relevant $[O_2]$ for the first time, and a large deuterium KIE was observed upon $k_{cat}/K_m(O_2)$. This result again indicates irreversible hydrogen transfer step after $O_2$ enters the catalytic cycle at physiologically relevant concentrations. Smaller deuterium KIEs at saturating [AA] and [O$_2$], i.e. $^{1}k_{cat}$, could arise from a change in hydrogen tunneling distance or in the kinetic mechanism. Comparing the temperature dependences of $^{D}k_{cat}$ and $^{D}k_{cat}/K_m(O_2)$ suggests a higher activation barrier required to attain the distance required for hydrogen tunneling from Tyr-371 to the enzyme-bound PGG$_2^\bullet$ derived from AA than from Tyr-371 to the smaller acyclic peroxyl radical derived from LA. Future studies of deuterium KIEs upon AA oxidation will address the origin of discrepancies in the temperature-dependent deuterium KIEs and mechanistic inconsistencies in the literature to date.

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