Oxidation Kinetics of Ethanol by Human Cytochrome P450 2E1
RATE-LIMITING PRODUCT RELEASE ACCOUNTS FOR EFFECTS OF ISOTOPIC HYDROGEN SUBSTITUTION AND CYTOCHROME b5, ON STEADY-STATE KINETICS

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A number of cytochrome P450 (P450) 2E1 substrates are known to show kinetic deuterium isotope effects of -5 on $K_m$ ($\Delta K = \frac{1}{2} \Delta K_m^0$), but not on $k_{cat}$, in rat liver microsomes (e.g. N-nitrosodimethylamine, ethanol, and CH$_2$Cl$_2$). We observed $0^5 K_m$ values of 3–5 for recombinant human P450 2E1-catalyzed ethanol oxidation. Replacing NADPH and O$_2$ with the oxygen surrogate cumene hydroperoxide yielded similar results. Ferric P450 2E1 reduction was fast ($k > 1000$ min$^{-1}$) even in the absence of substrate. These results indicate that the basis for the increase in $K_m$ is in the latter portion of the catalytic cycle. The intrinsic isotope effect ($\Delta K/k$) for ethanol oxidation was determined (competitively) to be $3,8$, indicating that C–H bond cleavage is isotopically sensitive. Pre-steady-state studies showed a burst of product formation ($k = 410$ min$^{-1}$), with the burst amplitude corresponding to the P450 concentration. Deuteration of ethanol resulted in an isotope effect of 3.2 on the rate of the burst. We conclude that product release is rate-limiting in the oxidation of ethanol to acetaldehyde by P450 2E1. The steady-state kinetics can be described by a paradigm in which the $k_{cat}$ approximates the rate of product release, and $K_m$ is an expression in which the denominator is dominated by the rate of C–H bond breaking.

Microsomal cytochromes P450 (also termed heme-thiolate protein P450 by the Enzyme Commission, EC 1.14.14.1) catalyze a variety of mixed-function monoxygenase reactions that often result in detoxication of drugs and other xenobiotics (2–4). Occasionally, oxidation results in the bioactivation of potentially potent carcinogens, particularly with substrates metabolized by P450 2E1 (5, 6). P450 2E1 is active in the oxidation of many low molecular weight organic compounds (e.g. nitrosamines and alkenes) associated with human cancers, and the reactivity of products with DNA has been demonstrated (7, 8). DNA alkylating ability and carcinogenicity were shown to be decreased upon deuterium substitution of N-nitrosodimethylamine, now known to be a substrate of P450 2E1 (9). When rat liver microsomes were examined, deuteration was found to increase $K_m$ for these reactions -5-fold, but the $k_{cat}$ ($V_{max}$) remained unaffected by deuterium substitution (10–12).

Most P450s are considered to operate according to a general scheme (Fig. 1) (13). Following substrate binding (step 1), ferric P450 receives 1 electron via NADPH-P450 reductase (step 2). Steps 1a and 2a represent a potential pathway by which Fe$^{3+}$ is reduced to Fe$^{2+}$ in the absence of substrate and suggests a possibility for later entry of the substrate into the catalytic cycle. The ferrous form of the heme binds O$_2$ (step 3) before undergoing a second 1-electron reduction to begin O$_2$ activation (step 4). Although this second electron originates from NADPH-P450 reductase, the accessory protein cytochrome $b_5$ ($b_5$; EC 4.4.2.4) appears to play some role in the delivery of the electron to the P450 (14). Insertion of the activated oxygen into the substrate is believed to occur by way of C–H bond cleavage (step 6) followed by rapid oxygen rebound to form product (step 7) (15). Step 8 is release of the product from the enzyme active site. Within the context of this scheme, the reduction (steps 2 and 4) and chemistry (step 6) are considered to be rate-limiting (15). Steps 9 and 10 reflect the potential for the Fe$^{2+}$ ROH complex to receive an electron from NADPH-P450 reductase, possibly leading to a second cycle of oxidation.

High intramolecular kinetic hydrogen isotope effects are seen in many P450 reactions involving C–H bond cleavage and are usually interpreted as evidence for a hydrogen atom abstraction mechanism (16), with some caveats (17). There are fewer reports of non-competitive intermolecular hydrogen isotope effects on P450 reactions, and these tend to be on $k_{cat}$ not $K_m$ (18).

Although the effects of deuteration substitution on N-nitrosodimethylamine N-demethylation were first reported in 1973, a definitive explanation for the intermolecular isotope effect on $K_m$ (and not $k_{cat}$) has not been given. The observation has been repeated using several P450 2E1 substrates (9–11, 19–22). Yang et al. (10) compared the competitive inhibition of alternate $^3$H and $^2$H substrates of P450 2E1 and suggested that the observed deuterium effect was due, in part, to a rate difference in the breaking of the C–H bond and its relationship with other rate constants. It was also proposed (10) that the effect on $V/K$ might be interpreted in the context of a generalized scheme (23, 24) in which (i) the isotopically sensitive step (C–H bond breaking) precedes a slower step (possibly product release) and (ii) the enzyme exhibits a low commitment to catalysis. Yang et al. (10) also suggested that rate-limiting product release could explain the results if a relatively high
degree of uncoupling of the activated P450-oxygen complex also occurred.

MMO is a mixed-function monoxygenase that is functionally similar to P450 2E1 (25). MMO requires a multi-enzyme complex and NADH as a co-substrate. The non-heme, two-iron catalytic center catalyzes the oxidation of a variety of low molecular weight hydrocarbons. Additionally, MMO has been shown to exhibit apparent isotope effects that are similar to those seen with P450 2E1. Chemical quench studies of the intermediates and interconversion rates in the MMO catalytic cycle revealed that product release is the rate-limiting step in this cycle (26, 27). Therefore, it is plausible that this might be the case for P450 2E1.

To determine the basis for the kinetic hydrogen isotope effects observed with P450 2E1, we have used deuterium substitution and pre-steady-state kinetic techniques to characterize the effect of deuteriation on individual steps of the catalytic cycle. The role of $b_5$ and its ability to enhance the rate of product formation were also examined. We interpret the observed kinetic isotope effects terms of rate-limiting product release and isotopically sensitive C–H bond cleavage.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Rabbit NADPH-P450 reductase (EC 1.6.2.4) and rabbit $b_5$ were purified as previously reported (28, 29). Recombinant human P450 2E1 was expressed in Escherichia coli and purified essentially as described (30). Recombinant NADPH-cytochrome P450 reductase was expressed in E. coli and purified by a modification of the method of Shen et al. (31). Apo-$b_5$ was prepared from rabbit liver $b_5$ by acid/acetone treatment (32).

**Chemicals**—Reagent grade ethanol was obtained from McCormick Distilling Co. (Weston, MO). [1-3H]Ethanol and [1-14C]ethanol were purchased from Cambridge Isotope Laboratories (Andover, MA). Lauric acid was purchased from Aldrich and recrystallized from CH3OH:H2O purchased from Cambridge Isotope Laboratories (Andover, MA). Pesticide grade CH3OH was from Burdick and Jackson (Muskegon, MI). Grade hexane was purchased from Mallinckrodt-Baker (Paris, KY), and [1-14C]lauric acid (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Before use, [1-3H]ethanol and [1-14C]ethanol were purified on Bakerbond™ octadecylsilane 3-ml disposable extraction columns (J. T. Baker Inc.) to remove, respectively, $^3$H$_2$O and [14C]acetalddehyde, by-products of radioactive ethanol synthesis. For purification, extraction columns were first washed with 10 ml of CH3OH and then equilibrated with 10 ml of H2O. [1-3H]Ethanol (100 μl diluted with H2O to 340 mM, 16.3 mCi mmol$^{-1}$) or [1-14C]lauric acid (170 mM, 10 mCi of mmol$^{-1}$) was loaded onto the column. Analytes were eluted with <3 ml of H2O, and 150-μl fractions were collected. A portion of each fraction was counted by liquid scintillation spectrometry. Radioactivity was plotted against fraction number, and the fractions comprising the major radioactive peak were pooled. The purity of the pooled solutions was confirmed by HPLC interfaced with radioflow detection (described under "Intrinsic Isotope Effect Estimation," see below). The concentrations of ethanol solutions were determined by measuring NADH formation spectrophotometrically at 340 nm in a reduction assay using yeast alcohol dehydrogenase (35). The purified solutions were then counted by liquid scintillation to determine the specific activity.

**Ethanol Oxidation Assay**—Unless otherwise specified, P450 2E1 (1.0 μM) was reconstituted with $b_5$ (2.0 μM), NADPH-P450 reductase (3.0 μM), and DLPC (30 μM) in 100 mM potassium phosphate buffer, pH 7.4. For steady-state measurements, 100-μl reactions were initiated with either an NADPH-generating system (36) or 1.0 mM cumene hydroperoxide and incubated for 10 min at 37 °C in reaction vials sealed with Teflon-lined rubber septa. Reactions supported by cumene hydroperoxide lacked NADPH-P450 reductase and $b_5$. Reactions were terminated with 20 μl of a mixture of 17% ZnSO$_4$ (w/v) and 0.55 mm semicarbazide (1:1, v/v) and centrifuged following addition of saturated BarOH (5.0 μl). The acetaldehyde product was derivatized to form the 2,4-dinitrophenylhydrazone (37) and then analyzed by HPLC using a Zorbax 6.2 × 80-mm octadecylsilane reversed-phase analytical column (3 μm, DuPont Chromatography Products, Wilmington, DE) (H2O:CH3CN, 45:55, v:v; 2.0 ml min$^{-1}$), monitoring A$_{340}$ (38). For reactions with [3H]- and [14C]ethanol, the HPLC peaks containing the radioactive product were collected and counted by liquid scintillation spectrometry, with calibration of counting efficiency using external [3H]- and [14C]acetate standards.

**Lauric Acid Hydroxylation Assay**—P450 2E1 (0.5 μM) was reconstituted with NADPH-P450 reductase and $b_5$ as described for the ethanol oxidation assay. Reactions (400 μl) with lauric acid or d$_{25}$-lauric acid (0–200 μM, as sodium salt) were terminated after 10 min at 37 °C by adding 50 μl of 12.5% H$_2$SO$_4$ (v:v). The quenched reactions were extracted twice with 6 ml of C$_2$H$_5$OH and the combined extracts were dried over Na$_2$SO$_4$ and then evaporated under a stream of N$_2$. The residue was redisolved in 100 μl of 18-crown-6-ether dissolved in CH$_3$CN (2.5 mg ml$^{-1}$), to which was added 100 μl of 4-bromomethyl-6,7-dimethoxycoumarin dissolved in (CH$_3$)$_2$CO (10 mg ml$^{-1}$) (in the presence of 2 mg of anhydrous K$_2$CO$_3$). The samples were incubated 60 min at 70 °C to form fluorescent derivatives. Product formation was measured by HPLC using a Zorbax 6.2 × 80 mm octadecylsilane re-
reversed-phase analytical column (3 μm, DuPont, H₂O:CH₃CN, 4:1, v:v; 2.0 ml min⁻¹). Fluorescence was monitored at λexcitation 375 nm, λmaxemission 470 nm (39). External standards of 11-hydroxylauric acid were used for quantitation. Experiments using [¹⁴C]lauric acid were quenched with ZnSO₄/semicarbazide mixture, and the combined extracts were dried under N₂, added aliquots were analyzed by HPLC interfaced with radioflow counting (Zorbax™ 4.6 × 250-mm octadecylsilane reversed-phase analytical column, 5 μm, DuPont) (H₂O:CH₃CN, 50:50, v:v; 2.0 ml min⁻¹). The [¹⁴C]11-hydroxylauric acid peak was collected and re-counted by liquid scintillation spectrometry.

Apparent Isotope Effect Determination—Deuterium isotope effects were determined by two methods (40). In a non-competitive method, P450 2E1 was incubated with d₄-ethanol or d₄-[1,1-³H]ethanol (0–100 mM) or d₄-lauric acid or d₄-[1,1-¹³C]lauric acid (0–150 μM), and the products were analyzed as described. Kₘ and kcat were calculated using a kcat nonlinear regression program (Bio-Metallics, Princeton, NJ). In a competitive method, P450 2E1 was incubated with a 1:1 mixture (v/v) of d₄-ethanol and d₄-ethanol (20 mM). The 2,4-dinitrophenylhydrazones were extracted into CH₂Cl₂ and analyzed by capillary column GC-NICIMS (45–47). Reactions with [¹⁴C]lauric acid in a 40-μl reaction volume for a period ranging from 2 ms to 10 min (as indicated) at 37 °C. Reactions with radioactive ethanol were quenched with a ZnSO₄/semicarbazide mixture and analyzed as described for steady-state reactions. Reactions using [¹³C]lauric acid were quenched by the addition of 30% pesticide-grade grade CH₃OH and derivatized with PFB-hydroxylamine by reacting the chlorzoxazone. The solutions were made anaerobic by alternating applications of vacuum and argon to a closed system as described previously (49). Reduction kinetics were monitored in a stopped-flow apparatus (model RQF-3, KinTek Corp., State College, PA). P450 2E1 (40 or 200 pmol) was incubated in the presence of either 200 mM [¹H]ethanol, 20 mM [¹³C]ethanol, or 100 μM [¹⁴C]lauric acid in a 40-μl reaction volume for a period ranging from 2 ms to 10 min (as indicated) at 37 °C. Reactions with radioactive ethanol were quenched with a ZnSO₄/semicarbazide mixture and analyzed as described for steady-state reactions. Reactions using [¹³C]lauric acid were quenched by the addition of 30% pesticide grade CH₃OH and derivatized with PFB-hydroxylamine by reacting the 250-μl quenched reaction volume with 20 μl of Florox™ reagent for 20 min at 60 °C. The oxime derivative was extracted into 1.0 ml of pesticide grade hexane, and the residual reagent was back-extracted following addition of 3 drops of concentrated H₂SO₄. The hexane layer (0.7 ml) was dried under N₂ and then redissolved in 20 μl of hexane. The oxime derivative was analyzed by GC-NICIMS (45–47). Reactions with [¹⁴C]lauric acid were analyzed as described previously.

NADPH Oxidation—P450 2E1 was reconstituted with NADPH-P450 reductase with or without b₅6 as described for steady-state ethanol oxidation experiments. Reconstituted enzyme (196 μM) was preincubated for 1 min at 37 °C in the presence or absence of substrate (20 mM ethanol or 0.15 mM chloroxazone). Reactions were initiated with the addition of 4 μl of 10 mM NADPH, and the decrease in absorbance at 340 nm was monitored spectrophotometrically. UV-visible spectra were recorded using a modified Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA). Rates of NADPH oxidation were calculated using ε₃₄₀ = 6.22 mm⁻¹ cm⁻¹ for NADPH.

H₂O₂ Formation—Reaction systems were prepared exactly as described above, except that reaction volumes were 0.5 ml. Reactions were initiated by adding the NADPH-generating system and were terminated by adding 0.8 ml of cold CF₃CO₂H (3%, w/v) after 10 min at 37 °C. H₂O₂ was determined spectrophotometrically by reaction with ferrozine and potassium thiocyanate as described (48).

Anaerobic Reduction Kinetics—Enzyme mixtures and NADPH (600 μM) solutions were prepared separately as described for steady-state reactions. Some enzyme mixtures contained 20 mM ethanol (or 0.15 mM chloroxazone). The solutions were made anaerobic by alternating applications of vacuum and argon to a closed system as described previously (49). Reduction kinetics were monitored in a stopped-flow apparatus (Applied Photophysics SX-17MV instrument, Applied Photophysics, Leatherhead, UK) at 37 °C under a CO atmosphere. Rapid reduction of P450 2E1 to a Fe²⁺-CO complex was observed at 450
nm upon mixing of reconstituted enzymes with NADPH. Data were collected using the Applied Photophysics software system and fitted to exponential equations using a Marquardt-Levenberg algorithm for non-linear regression analysis. Results are reported as three to eight individually monitored reactions averaged using the manufacturer's software.

**Mass Spectrometry**—The hydrazone derivatives were analyzed by GC-PIEIMS. Analytes were separated on a 15-m SPB<sup>TM</sup>-1 fused silica capillary column (Supelco, Bellefonte, PA) interfaced to a Finnigan INCOS 50 mass spectrometer (Finnigan, San Jose, CA). GC conditions were as follows: carrier gas (2He) at constant pressure of 10 p.s.i.; injection port 230 °C; transfer line 260 °C. The initial column temperature was 150 °C and was increased to 300 °C at a rate of 20 °C min<sup>−1</sup>. The [1H]acetaldehyde hydrazone was detected by selected ion monitoring at m/z 224, and the [2H]acetaldehyde hydrazone was monitored at m/z 225 (Fig. 4). GC-NICIMS of the PFB-oxime derivatives was performed using a 30-m SPB<sup>TM</sup>-5 fused silica capillary column (Supelco) coupled to a Hewlett-Packard 5989A mass spectrometer (Hewlett-Packard, Wilmington, DE). GC conditions were as follows: carrier gas (2He) at constant pressure of 0.8 p.s.i.; injection port 265 °C; transfer line 270 °C. The column was initially held at 65 °C for 10 min, increased by 5 °C min<sup>−1</sup> to 100 °C, and then raised at 20 °C min<sup>−1</sup> to 250 °C (Fig. 5). Acetaldehyde-PFB oxime was quantitated by selected ion monitoring at m/z 181, using propionaldehyde-PFB oxime as an internal standard. The retention times of the analytes were verified by monitoring ions corresponding to M<sup>+</sup> – 20 (M<sup>+</sup>–HF) for each of the oximes (Fig. 6).

**RESULTS**

**Steady-state Kinetic Parameters**—In rat liver microsomes, deuterium substitution of P450 2E1 substrates usually results in a 3–5-fold increase in K<sub>m</sub> without any effect on k<sub>cat</sub> (9, 11, 12, 19, 20, 50). Recombinant human cytochrome P450 2E1 was reconstituted with NADPH-P450 reductase and b<sub>5</sub> and incubated with a variety of substrates and their deuterated analogs to determine the effects of deuterium substitution on the steady-state kinetic parameters (Fig. 7 and Table I). Deuteration of ethanol increased the K<sub>m</sub> 4.7-fold. k<sub>cat</sub> was essentially unaffected so that D<sup>V</sup> ≈ 1 (Fig. 7A). In a reaction system supported by the oxygen surrogate cumene hydroperoxide, the effects on K<sub>m</sub> and k<sub>cat</sub> for ethanol oxidation were the same. K<sub>m</sub> was increased 6.0-fold and k<sub>cat</sub> was unchanged. For each of these systems, the apparent deuterium isotope effect, D<sup>V/K</sup>, was ~5.<sup>2</sup>

<sup>2</sup>In subsequent experiments presented here, catalytic rates were generally higher with other preparations of P450 2E1 and NADPH-P450 reductase under more refined conditions. However, the apparent deuterium isotope effect was similar (~3–5) for all preparations.
Cumene and remained unaffected.

Reductase, Reductase, [1H]ethanol or 0–100 mM [2H]ethanol or with 0 to 150 mM [1H]ethanol or 0 to 150 mM [2H]ethanol or with 0 to 25 mM [1H]ethanol or 0–100 mM [2H]ethanol or with 0 to 150 mM [1H]- or [2H]-lauric acid. Kinetic constants are shown in Table I. Results are shown as the average of duplicate rate (v) determinations for each concentration of substrate. Fits were done using non-linear regression analysis. Rates were expressed as nmol product min⁻¹ (nmol P450 2E1)⁻¹.

TABLE I
Steady-state kinetic parameters for oxidations catalyzed by recombinant human cytochrome P450 2E1

| System                      | $k_{cat}$ (nmol min⁻¹/nmol P450) | $K_m$ (mM) | $\Delta V/K_{cat}$ (nmol/min/nmol P450)⁻¹ | $V/K_{cat}$ (mM) |
|-----------------------------|---------------------------------|------------|------------------------------------------|------------------|
| Reduced, b₅                  | 2.7 (±0.21)                     | 11.2 (±1.6) |                                           |                  |
| CH₃CH₂OH                    | 2.4 (±0.14)                     | 52.6 (±6.4) | 1.1                                      | 5.2              |
| Reductase, b₅               | 0.81 (±0.07)                    | 5.3 (±1.2)  |                                           |                  |
| CH₃CD₂OH                    |                                |            |                                          |                  |
| Cumene hydroperoxide, CH₃CH₂OH| 1.02 (±0.05)                   | 32 (±4)    | 0.79                                      | 4.7              |
| Cumene hydroperoxide, CH₃CD₂OH|                            |            |                                          |                  |
| Reductase, b₅, lauric acid  | 3.2 (±0.5)                      | 0.066 (±0.024) |                                           |                  |
| Reductase, b₅, d₁₂-lauric acid| 1.5 (±0.3)                     | 0.061 (±0.020) | 2.1                                      | 2.0              |

For lauric acid 11-hydroxylation, the isotope effect was on $k_{cat}$ rather than on $K_m$ (Fig. 7B). Deuteration of lauric acid decreased $k_{cat}$ from 2.4 to 1.5 min⁻¹ ($\Delta V = 2.1$), whereas $K_m$ remained unaffected.

Estimation of Intrinsic Isotope Effect ($Dk$) — $Dk$ for ethanol oxidation was determined from the Swain relationship, which considers $V/K_{cat}$ as well as the apparent tritium isotope effect, $V/K_{cat}$. The approach has been applied to another P450-mediated reaction, 7-ethoxytocumarin O-deethylation, by Miwa et al. (41), and an identical strategy was used here. $V/K_{cat}$ was measured in a competitive assay in which P450 2E1 was incubated with a 1:1 mixture of d₅- and d₄-ethanol. $V/K_{cat}$ was calculated as a ratio of $\text{[H]}$ to $\text{H}$ removal from the substrate and was found to be 3.9 ± 0.3. $V/K_{cat}$ was also determined competitively by incubating the enzyme with a mixture of [1H]- and [1-3H]ethanol. Removal of $^3$H from the C1 position results in the formation of a molecule of $^3$H₂O. Alternatively, removal of $^3$H from [1-3H]ethanol results in the formation of [1-3H]acetaldheyde. Using the equation $\tau(V/K) = (\log(1 - f)/\log(1 - f/SA_{d}/SA_{e}))$ (defined under “Experimental Procedures”), $\tau(V/K) = 6.8$. From the relationship $(Dk - 1)/(Dk^{1.442} - 1) = (V/K_{cat})^{-1}/(\tau(V/K) - 1)$, $Dk = 3.8$.

Effect of $b₅$ on Steady-state Kinetic Parameters — $b₅$ has been shown to enhance the rate of product formation in P450 2E1-catalyzed reactions (38, 51–54). When P450 2E1 was reconstituted with or without $b₅$, the values for $k_{cat}$ were 16 and 19 min⁻¹, respectively (data not shown). The $K_m$ for ethanol was increased ~2-fold, from 28 to 52 mM, in the absence of $b₅$. Apo-$b₅$ did not have an effect. The effect of deuterium substitution on ethanol oxidation in the reactions system lacking $b₅$ was the same as observed in the reaction system that included $b₅$.

Anaerobic Reduction of Ferric P450 2E1 — To evaluate the
role of substrate on P450 2E1 reduction rates, P450 2E1 was reconstituted with NADPH-P450 reductase, and reduction was monitored in the presence or absence of 170 mM ethanol (Fig. 8). P450 2E1 showed biphasic reduction kinetics that could be fit to bi-exponential equations to calculate rate constants. P450 2E1 reduction rates were not substantially altered by adding substrate to the reaction mixture. The rapid phase of reduction was 1800 min\(^{-1}\) without ethanol and 1380 min\(^{-1}\) with ethanol. The rates of the slow phase were 120 and 300 min\(^{-1}\) with and without ethanol, respectively.

\( b_5 \) decreased the apparent rate of reduction of ferric P450 2E1 from 1800 to 420 min\(^{-1}\) (rapid phase) and from 300 to 12 min\(^{-1}\) (slow phase). Further addition of ethanol to the reaction system already containing \( b_5 \) did not significantly change the reduction rate (results not shown).

**Uncoupling of P450 2E1 Reactions**—The effects of \( b_5 \) on NADPH oxidation and \( O_2 \) utilization were also examined. P450 2E1 reconstituted with \( b_5 \) showed a 35% decrease in NADPH oxidation rates (from 55 to 36 min\(^{-1}\)) as compared with P450 2E1 systems that lacked \( b_5 \). \( H_2O_2 \) formation remained relatively unchanged (14 min\(^{-1}\)), \( b_5 \) yielded a 42% increase in the rate of acetaldehyde formation, indicating more efficient coupling, consonant with results of others with P450 2E1 (55).

**Pre-steady-state Kinetics and Rate-limiting Product Release**—Rapid quench kinetic techniques were used to determine pre-steady-state product formation in P450 2E1-catalyzed ethanol oxidation. Initially, \( ^3H \) and \( ^14C \)-radiolabeled substrates were used to improve sensitivity for detection of product formation (Fig. 9, A and B). Although substrates were purified before use, the remaining background contribution confounded determination of the burst phase amplitude for oxidation of \( ^14C \) alcohol (Fig. 9B). An improved method of quantitation involved formation of the PFB-oxime derivative of acetaldehyde, which could be analyzed by GC-NICIMS (Fig. 9C). This system also has the advantage that stable isotopes may be utilized.

All three methods clearly revealed two distinct phases of product formation for ethanol oxidation. In each case, a rapid pre-steady-state burst of product formation was followed by a much slower phase of product formation that roughly corresponded to the observed steady-state rate. The rates of burst phase product formation were 410, 370, and 410 min\(^{-1}\) for \( ^3H \)-, \( ^14C \)-, and \( ^1H \)-ethanol, respectively (Fig. 10). The amplitudes of the bursts correlated well with the amounts of P450 used in the reaction.

Pre-steady-state analysis of product formation during lauric acid hydroxylation did not show a kinetic burst. The time course for product formation was linear through the first turnover cycle and into subsequent cycles (Fig. 9D).

**Effects of \( b_5 \) and \( ^2H \) Substitution on Burst Phase Product Formation**—Since \( b_5 \) has been shown to enhance both the rate of product formation and the extent of \( O_2 \) coupling in steady-state product formation, its effect on pre-steady-state product formation was examined. The amplitude of the burst was diminished in comparison to reactions containing \( b_5 \) (Fig. 10B). Only \(-30\%\) of P450 yielded product during the burst. The rate of the burst, however, was similar to the rates observed with \( b_5 \) present.

Although deuterium substitution does not affect steady-state rates of product formation at high substrate concentration, the measurable intrinsic isotope effect suggests that subsets of reaction steps may be affected if they include the isotopically sensitive step. Deuteration of ethanol decreased the rate of the burst phase from 410 to 130 min\(^{-1}\), so that \( ^2V \) for burst phase product formation is 3.2 (the experiment was done at saturat-
Independent of substrate (56–58). Following oxygen activation, as the catalytic cycle (as described in Fig. 1). Additionally, early steps, i.e., ferric P450 2E1 reduction (step 2 or 1a) and oxygen activation (steps 3–5), were not affected by substrate. The effect on the rate-limiting step of Fig. 1 should be preferred to explain the results, and the MMO model appears more reasonable.

$b_n$, which has been shown to enhance the product formation rates with a number of substrates for P450 2E1 (i.e. chlorozoxone, N-nitrosodimethylamine, aniline, diethyl ether) (38, 53, 54), did not alter the deuterium effect on $V/K$ for ethanol oxidation but did slow the rates of steps 1a and 2 and decrease steady-state NADPH oxidation, in agreement with previous reports (55). The effect of $b_n$ on apparent P450 reduction rates may be explained in a model in which electrons from NADPH-P450 reductase are transferred to P450 2E1, which then reduces $b_n$ and requires reduction again (59).

Rapid quench kinetic experiments clearly indicated a rapid burst of pre-steady-state product formation ($k = 400 \text{min}^{-1}$) for ethanol oxidation. This behavior is indicative of a mechanism in which the rate-limiting event occurs after product formation. We assign the rate-limiting step as product release.

The isotope effect on $K_m$, but not on $k_{cat}$, cannot be explained in terms of a model where $K_m \sim k_{cat} = (k_{-1} + k_2)/k_1$, where $k_2$ is a single slow step approximating $k_{cat}$ and both $k_1$ and $k_{-1}$ (substrate binding and dissociation) are rapid. The only isotopically sensitive step in P450 catalysis should be step 6 of Fig. 1. We propose that in the optimal reconstituted P450 2E1 system (with NADPH-P450 reductase and $b_n$), or in microsomes, product release is effectively rate-limiting. Thus (using the nomenclature of the steps in Fig. 1) $k_{cat} \sim k_8 [E]_T$ and (see Equation 3)

$$K_m \sim \frac{k_{-1}}{k_8} \quad (\text{Eq. 3})$$

(where $[E]_T$ is the total enzyme concentration). However, the units for the approximation of $K_m$ are inappropriate, and no dependence on $[RH]$, the concentration of substrate, would be expected. A more extensive treatment of the system is available in a model presented by Kuby (60) and suggested for consideration in our own earlier review of P450 isotope effects (61). In this case, which is still simplified, the reaction is depicted as shown in Equation 4.

$$ES \rightarrow EP \rightarrow E + P \quad (\text{Eq. 4})$$

where $S$ is the substrate and $P$ the product. Then

$$k_{cat} = \frac{k_5 S}{k_2} \quad (\text{Eq. 5})$$

$$K_m = \frac{k_5 S}{k_3 + k_5} \quad (\text{Eq. 6})$$

so that $K_m$ has units of molarity. Therefore,

$$D^V = \frac{D^V}{1 + k_5/k_3} \quad (\text{Eq. 7})$$

and substrate undergoes a first-order reaction with activated oxygen (flavin 4a-hydroperoxide) to form product. Andersen et al. (50) proposed a similar mechanism for P450 2E1 that involved rate-limiting $O_2$ activation preceding substrate binding. We are of the opinion that bacterial MMO (25–27) serves as a better model for the observations with P450 2E1. The catalytic center of MMO can be reduced prior to substrate binding. Additionally, MMO shows kinetic hydrogen isotope effects similar to P450 2E1, and product release has been proven to be rate-limiting in the MMO catalytic cycle (26, 27). Although a mechanism resembling that for flavin-containing monooxygenases cannot be dismissed, there is no strong evidence that this should be preferred to explain the results, and the MMO model appears more reasonable.

**Fig. 10.** Burst analysis of pre-steady-state product formation in P450 2E1-catalyzed ethanol oxidations. A, the experiment is as described in Fig. 9C. B and C, the experiment is the same except that $b_n$ is absent (B) or [1,1-$^2$H]ethanol is replaced with [1,1-$^2$H]ethanol (C). The amount of P450 2E1 in each experiment is 40 pmol.

**Discussion**

Isotopic substitution is frequently used as a probe for studying enzyme mechanisms. In reactions that involve a proton transfer step, deuterium of substrates often produces an apparent isotope effect on $k_{cat}$ ($V_{max}$). Oxidation of substrates by P450 2E1 involves a C–H bond breaking step, but deuterium substitution has been shown by several investigators to have little effect on $k_{cat}$. Rather, there is an intermolecular isotope effect on $K_m$ for many substrates of P450 2E1 (9, 11, 12, 19, 20, 50).

In liver microsomes, this isotope effect on $V/K$ (but not $k_{cat}$) has been reported for $N$-nitrosodimethylamine (10, 12, 54), CH$_2$Cl$_2$ (50), and ethanol (10). We measured $V/K$ values of 3–5 for ethanol oxidation catalyzed by purified recombinant human P450 2E1. In a system where NADPH and $O_2$ were replaced with the oxygen surrogate cumene hydroperoxide, similar isotope effects were observed, indicating that the basis for the effect on $K_m$ is related to steps in the latter portion of the catalytic cycle (as described in Fig. 1). Additionally, early steps, i.e. ferric P450 2E1 reduction (step 2 or 1a) and oxygen activation (steps 3–5), were not affected by substrate. Enzyme reduction prior to the addition of substrate is reminiscent of the mechanism of liver microsomal flavin-containing monooxygenases in which FAD reduction by NADPH is preceded by and is independent of substrate (56–58). Following oxygen activation, and

$$k_{cat} = \frac{k_5 S}{k_2} \quad (\text{Eq. 5})$$

$$K_m = \frac{k_5 S}{k_3 + k_5} \quad (\text{Eq. 6})$$

and

$$D^V = \frac{D^V}{1 + k_5/k_3} \quad (\text{Eq. 7})$$

and
of the oxygen in the form of H$_2$O$_2$ or H$_2$O. This is supported by the high level of coupling seen in the pre-steady-state phase of product formation preceding rate-limiting product release.

It is clear that this model is not valid for all substrates for P450 2E1. As with lauric acid, deuterium substitution of the substrate 7-ethoxycoumarin resulted in a decrease in $k_{cat}$ with no effect on $K_m$, when 7-hydroxycoumarin was measured as the product (results not shown). Whether this is a result of switching to alternate pathways (65–68) or formation of a product that does not show rate-limiting product release remains to be investigated. However, our current view is that the paradigm of rate-limiting product release should explain all P450 2E1-catalyzed reactions in which a kinetic hydrogen isotope effect on $K_m$ has been observed (9–12, 19, 20, 50).

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