The P450 2E1-catalyzed oxidation of ethanol to acetaldehyde is characterized by a kinetic deuterium isotope effect that increases \( K_m \) with no effect on \( k_{cat} \) and rate-limiting product release has been proposed to account for the lack of an isotope effect on \( k_{cat} \) (Bell, L. C., and Guengerich, F. P. (1997) J. Biol. Chem. 272, 29643–29651). Acetaldehyde is also a substrate for P450 2E1 oxidation to acetic acid, and \( k_{cat}/K_m \) for this reaction is at least 1 order of magnitude greater than that for ethanol oxidation to acetaldehyde. Acetic acid accounts for 90% of the products generated from ethanol in a 10-min reaction, and the contribution of this second oxidation has been overlooked in many previous studies. The non-competitive intermolecular kinetic hydrogen isotope effects on acetaldehyde oxidation to acetic acid \( (1/2k_{cat}/K_m)^2(k_{cat}/K_m) = 4.5 \), and \( 1/2k_{cat} = 1.5 \) are comparable with the isotope effects typically observed for ethanol oxidation to acetaldehyde, and \( k_{cat} \) is similar for both reactions, suggesting a possible common catalytic mechanism. Rapid quench kinetic experiments indicate that acetic acid is formed rapidly from added acetaldehyde \((-450 \text{ min}^{-1})\) with burst kinetics. Pulse-chase experiments reveal that, at a saturating concentration of ethanol, \( \sim 90\% \) of the acetaldehyde intermediate is directly converted to acetic acid without dissociation from the enzyme active site. Competition experiments suggest that P450 2E1 binds acetic acid and acetaldehyde with relatively high \( K_m \) values, which preclude simple tight binding as an explanation for rate-limiting product release. The existence of a rate-determining step between product formation and release is postulated. Also proposed is a conformational change in P450 2E1, which may be a rate-determining step between product formation and release is postulated. The microsomal cytochrome P450 enzymes are the major enzymes involved in the oxidation of xenobiotic chemicals in eukaryotes (3–5). The P450 monooxygenases catalyze a multitude of oxidation reactions, such as hydroxylation of aliphatic and aromatic carbons, epoxidation of olefins, \( N \)-dealkylation of amines, and \( O \)-dealkylation of ethers (5, 6). Although a few general catalytic mechanisms appear to be operative for most of the reactions catalyzed by the P450s (Fig. 1) (7–9), certain features such as rate-determining steps and substrate interactions can vary considerably (8, 11, 12).

P450 2E1 is considered to be one of the major human hepatic P450 enzymes (13). Human P450 2E1, as well as the animal orthologs, accepts a broad range of substrates, with apparent preference for small and hydrophobic molecules (4, 14, 15). P450 2E1 is notably active in the oxidation of many low \( M_r \) volatile solvents with common industrial applications and issues of cancer risk (16, 17).

Many P450 2E1 reactions have been characterized, at least in terms of the products (17, 18), and the Michaelis-Menten constants have been determined. Some reactions have been further studied by employing isotopic substitution of the substrate. Deuteration of several P450 2E1 substrates results in a 3–5-fold decrease in \( k_{cat}/K_m \) without any effect on \( k_{cat} \) (19). One example is the P450 2E1-catalyzed oxidation of ethanol to acetaldehyde (10, 19). This laboratory, as well as others, has indicated that this kinetic hydrogen isotope effect can be explained by rate-limiting product release following the isotopically sensitive and essentially irreversible C–H bond-breaking step (19, 20), and experimental evidence has been offered in support of this model (10). A carbonyl product is generated in each of the oxidation reactions displaying this pattern of isotope effects (19, 21–24).

Kunitoh et al. (25) and Terelius et al. (26) have previously reported that acetaldehyde, generated from ethanol oxidation, serves as a substrate for P450 2E1 and is oxidized to acetic acid. We have used steady-state, rapid quench, and pulse-chase kinetic experiments to analyze the sequential oxidations that convert ethanol to acetaldehyde and, ultimately, to acetic acid. KINSIM and FITSIM computer simulations are presented for ethanol oxidation by P450 2E1, and a sequential oxidation mechanism involving a rate-determining step between product formation and release is postulated.

**MATERIALS AND METHODS**

**Enzymes**—Rabbit NADPH-P450 reductase and rabbit cytochrome \( b_5 \) were purified as previously reported (27–29). Recombinant human P450 2E1 was expressed in *Escherichia coli* and purified essentially as described (15). *Saccharomyces cerevisiae* pyruvate decarboxylase, stabilized with thiamine pyrophosphate, was purchased from Sigma and dialyzed at 4 °C against 3 × 500-fold volumes of 50 mm potassium phosphate buffer (pH 6.5) in \( \text{H}_2\text{O} \) (Aldrich; 99.9% atomic excess) prior to use.

**Conventions**—Prior to use. \( V/V(K) = \frac{k_{cat}}{K_m} \) and \( 1/(V/K) = \frac{k_{cat}}{K_m} \).
to use in the synthesis of [1-^14^C]acetdehyde.

**Chemicals**—Acetaldehyde, acetic acid, and 4-methylpyrazole were purchased from Aldrich; acetaldehyde was purified by distillation at atmospheric pressure prior to use. Stock solutions of 50 mM 4-methylpyrazole in H$_2$O were used in binding studies. Reagent grade ethanol was obtained from J.T. Baker, Phillipsburg, NJ) to remove components that might interfere with [1^14^C]acetic acid and [1^14^C]acetdehyde determinations (10). P450 2E1 (10 mM, 3^14^C/formaldehyde (55 mM $\mu$M $^{-1}$) were purchased from American Radiolabeled Chemicals (St. Louis, MO); [1^14^C]acetdehyde was purified prior to use by passing it over a Bakerbond™ quaternary amine 3-ml disposable SPE column. Dinitrophenylhydrazine-HCl (Eastman Kodak Co.), was recrystallized from H$_2$O before use.

**Synthesis of [1-^14^C]Acetdehyde—[1-^14^C]Acetdehyde was synthesized using pyruvate decarboxylase, an enzyme that converts pyruvic acid to acetaldehyde with the addition of a solvent proton (retained at the aldehyde C-1 position) (30). All reagents were dissolved in H$_2$O and purged with argon. The reaction solution contained 10 units of pyruvate decarboxylase (dialyzed versus H$_2$O as described above), 1.0 mM thiamine pyrophosphate, 1.6 mM MnSO$_4$, 15 mM [2-^14^C]pyruvic acid (50 mCi, 16.6 mCi mmol$^{-1}$), NEN Life Science Products (DuPont) (H$_2$O:CH$_3$CN, 45:55, v:v; flow rate 2.0 ml min$^{-1}$), monitoring $A_{210}$ (35). Acetic acid generated from [1-^14^C]ethanol was quantitated as described above.

**Apparent Isotope Effect Determination**—Deuterium isotope effects were determined by a noncompetitive method (2). P450 2E1 was incubated with unlabeled (d$_0$) acetaldehyde or [1-^14^C]acetdehyde (d$_1$), and the products were analyzed as described. $K_p$ and $K_{cat}$ were calculated using the kcat nonlinear regression program (Bio-Metallics, Princeton, NJ).

**Pre-steady-state Kinetics**—Pre-steady-state acetdehyde oxidation reactions were done in a quench-flow apparatus (model RQF-3, KinTek Corp., State College, PA). P450 2E1 (50 pmol) was reconstituted as described and incubated with the presence of 200 $\mu$M [1-^14^C]acetdehyde in a total volume of 25 $\mu$l (per injection). Reactions were initiated by rapid mixing with 25 $\mu$l of 1.0 mM NADPH (50-$\mu$l total volume) for a period of time ranging from 2 ms to 2 min (as indicated) at 37°C. In a similar reaction, ethanol oxidation was initiated by the addition of ethanol to reconstituted P450 2E1 (10). Reactions were quenched with 2.0% ZnSO$_4$ (w/v), and acetic acid was quantitated as described for steady-state reactions.

**Kinetic Pulse-Chase Experiments**—Kinetic pulse-chase experiments were conducted in a manner similar to the pre-steady-state experiments. Reconstituted P450 2E1 (50 pmol), preincubated with 10 or 30 mM [1-^14^C]ethanol (reaction concentrations were 5 and 15 mM [1-^14^C]ethanol, respectively), was rapidly mixed (using a quench-flow apparatus) with an equal volume of 1.0 mM NADPH. Reactions proceeded for 100 ms prior to being chased with a 5-fold volume excess of either H$_2$O or 3 mM unlabeled acetaldehyde. Chasing continued for an additional 10 s before being quenched with 10 $\mu$l of 2.0% ZnSO$_4$ (w/v). The [1^14^C]acetic acid product was analyzed as described using Bakerbond™ quaternary amine 3-ml SPE columns. Evidence of dissociation of the intermediate, acetaldehyde, was observed as dilution of specific activity in the product (acetic acid).

**Enzyme Constant of Binding Constants**—P450 2E1 (1.0 $\mu$m) in 100 mM potassium phosphate buffer (pH 7.4, 2.0-ml total volume) was divided equally between two cuvettes, and a base-line spectrum was acquired from 340 to 480 nm using an OLIS-Amino DW2a spectrophotometer (On-Line Instrument Systems, Bogart, GA). To the sample cuvette, 0.3 or 3.0 $\mu$m 4-methylpyrazole was added, and the resulting “Type II” binding spectrum was recorded and designated as “100% binding.” Aqueous potassium acetate (pH 7.4, 0–35 mm) or acetaldehyde (0–15 mm) was titrated into both the sample and reference cuvettes, and disappearance of the 4-methylpyrazole-induced Type II binding spectrum was quantified on the basis of the decrease in the absorbance difference ($\Delta A$) between 424 and 389 nm ($\Delta A = A_{424} - A_{389}$).

**Kinetic Simulations by Computer Modeling**—Simulations modeling the observed kinetic data for ethanol oxidation by P450 2E1 were done by a Monte Carlo analysis of FTSIM (version 3.1, 1997 version), obtained from Prof. C. Frieden (Washington University, St. Louis, MO). (36). Regression analysis was performed using the companion program FITSIM (37). Both programs were run in DOS version 7.0 on an IBM personal computer. Final fitting was done using FITSIM. Iterative nonlinear regression analysis of the curves is useful for further refinement of kinetic constants and for overall evaluation of the kinetic model based on the fit of the simulation to the experimental data.

**RESULTS**

**Steady-state Kinetic Parameters**—For several oxidations catalyzed by P450 2E1, deuterium substitution of the substrate produces an apparent isotope effect on $V/K$ but not on $V$ ($k_{cat}$). Ethanol is oxidized to acetaldehyde by this P450, and rate-limiting product release has been proposed for this reaction (10, 19). Acetaldehyde, the product of one such reaction characterized by this pattern of isotope effects, has been reported to be a substrate for P450 2E1 (25, 26). Acetaldehyde was oxidized by P450 2E1 with a value of $k_{cat}/K_p$ at least 1 order of magnitude greater than for ethanol oxidation to acetaldehyde (Table I). The $K_p$ parameter for the oxidation of acetaldehyde showed more variability than other parameters. For instance, a different preparation of P450 2E1 (than the one used in Table I) yielded $k_{cat} = 8.6 \pm 0.8$ min$^{-1}$ and $K_p = 22 \pm 3$ mM for the oxidation of ethanol to acetaldehyde and $k_{cat} = 8.2 \pm 0.8$ min$^{-1}$ and $K_p = 86 \pm 7$ $\mu$m for the oxidation of acetaldehyde to acetic acid, for an enzyme efficiency $(V/K)$ 240-fold better for the latter reaction. In all work presented in the rest of this paper, direct
isotope effect on acetic acid was measured (Fig. 2). By contrast, only a minimal isotope effect was observed within the enzyme, stoichiometric with the amount of P450 2E1, and much slower phase of product formation, occurring at a rate constant of 0.01 s⁻¹. However, it was necessary to follow rapidly quench kinetic studies demonstrated a rapid aldehyde oxidation to acetic acid, it was necessary to follow for rate-determining product release within the context of acetyl-CoA. Thus, we have now characterized the P450 2E1-catalyzed oxidation of acetaldehyde to acetic acid in reactions containing 5 and 15 mM [¹⁴C]ethanol (Table II).

**DISCUSSION**

In a previous study, we characterized the P450 2E1-catalyzed oxidation of ethanol to acetaldehyde and rationalized the observed kinetic deuterium effect on $K_m$ in terms of an intrinsic isotope effect on C–H bond breaking and burst kinetics, interpreted to result from a (steady-state) rate-determining step following product release (10). We have now characterized the P450 2E1-catalyzed oxidation of acetaldehyde to acetic acid and also find burst kinetics and a kinetic deuterium isotope effect on $K_m$ (but not $k_{cat}$). A simplified interpretation of this reaction is that developed for ethanol oxidation (10), where (using the steps labeled in Fig. 1)

$$k_{cat} \sim k_E [E]_{T}$$

(Eq. 1)

and

$$K_m = \frac{k_S}{k_E}$$

(Eq. 2)

(9)

where $[E]_T$ is the total enzyme concentration). A more general description of $K_m$ is provided with the simplified model of Kuby (39),

$$k_S E \equiv ES \rightarrow E + P \rightarrow E + P$$

(Eq. 3)

where $S$ is the substrate acetaldehyde and $P$ is the product acetic acid (10). As before (10, 39),

$$k_{cat} = \frac{k_S E}{k_E}$$

(Eq. 4)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
<th>$V/K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃CH₂OH → CH₃CHO</td>
<td>12.6 ± 0.4</td>
<td>10.4 ± ± 0.12</td>
<td>1.2</td>
</tr>
<tr>
<td>CH₃CHO → CH₃COOH</td>
<td>7.6 ± 0.2</td>
<td>0.50 ± 0.03</td>
<td>15</td>
</tr>
<tr>
<td>CH₃CH₂OH → CH₃COOH</td>
<td>6.0 ± 0.8</td>
<td>0.84 ± 0.42</td>
<td>7.1</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>[¹⁴C]Ethanol (present during 100-ms pulse)</th>
<th>[¹⁴C]Acetic acid formed</th>
<th>[¹⁴C]Acetic acid remaining after chase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without 20 mM acetaldehyde chase</td>
<td>0.32 ± 0.03</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>With 20 mM acetaldehyde chase</td>
<td>1.58 ± 0.10</td>
<td>1.41 ± 0.20</td>
</tr>
</tbody>
</table>

**Table I Summary and comparison of steady-state kinetic constants for reactions catalyzed by P450 2E1**

$k_{cat}$ and $K_m$ were calculated using the program kcat. All parameters were determined with a single set of enzymes. The indicated variability was estimated by the software program.
**Fig. 1. General scheme for P450-catalyzed oxidation reactions. RH, substrate; ROH, product. The reversibility of some of the latter steps is unknown.** Outlets for the uncoupled reduced oxygen products O₃, H₂O₂, and H₂O are shown. As pointed out earlier (10), with P450 2E1 the binding of substrate can follow step 2 and possibly 3 and 4.

\[
K_m = \frac{k_1(k_2 + k_3)}{k_1(b_3 + b_3)} \quad \text{(Eq. 5)}
\]

which can be reduced to

\[
k_{cat} = k_{3}[E_I] \quad \text{(Eq. 6)}
\]

and

\[
K_m = \frac{k_2k_3}{k_2b_3} \quad \text{(Eq. 7)}
\]

and attenuation of \(k_3\) (Equation 7) due to hydrogen substitution can raise \(K_m\) (10).²

The P450 2E1-catalyzed conversion of ethanol to acetic acid was studied as an example of a multistep oxidation pathway. The time course of the reaction (at low ethanol concentration) was characterized by a lag in the formation of acetic acid, a low steady-state level of acetaldehyde, and the recovery of a large fraction of the total product as acetic acid after 10 min at 37 °C (Fig. 3). Pulse-chase experiments, patterned after the work of others (41, 42), indicate that most of the acetaldehyde formed does not dissociate readily from the enzyme and equilibrate with a pool of acetaldehyde in the medium (Table II).

Several P450 enzymes catalyze multistep enzyme reactions. Of particular physiological relevance are several involved in steroid oxidations, e.g. P450s 11B, 17A, 19, 24, 27, and 51 (41–45). These reactions usually consist of three consecutive two-electron oxidation steps. Most are considered to involve little exchange of the reaction intermediates with the medium, as indicated by pulse-chase experiments (Table II). The P450 2E1-catalyzed conversion of ethanol to acetic acid is shown in Fig. 5. Several points can be made regarding fits to this and other models (Fig. 5) as follows. (i) \(K_m\) values for binding of P450 2E1 and either ethanol, acetaldehyde, or acetic acid are all high (millimolar to molar range). (ii) In the oxidation of ethanol to acetaldehyde, the C–H bond breaking step is rapid (>500 min⁻¹), based on pre-steady-state results, and there is low commitment to catalysis up to and including this step, as demonstrated by the high \(V/K\) values (10). (iii) Ethanol binds rapidly to P450 2E1, since burst kinetics are not attenuated when P450 2E1 and ethanol are mixed from separate syringes (10). (iv) Some step following acetaldehyde formation is slow (in the oxidation of ethanol to acetaldehyde), because of the burst kinetics (10). (v) Acetaldehyde bound to P450 2E1 does not readily equilibrate with the medium, as indicated by pulse-chase experiments (Table II). (vi) Oxidation of acetaldehyde to acetic acid is accompanied by high \(V/K\) and \(V^1\) values similar to those found for ethanol oxidation to acetaldehyde (Fig. 2) and is postulated to involve similar explanations, at least when the reaction involves the direct addition of acetaldehyde to P450 2E1 (see above). (vii) The chemistry step (involving C–H bond breaking) in the oxidation of acetaldehyde to acetic acid is rapid, as indicated by the (stoichiometric) burst kinetics (Fig. 5). (viii) Binding of acetaldehyde to (oxidized) P450 2E1 is rapid, since burst kinetics were also observed when acetaldehyde was added to P450 2E1 from a separate syringe (results not shown).

A minimal KINSIM model for the overall scheme of ethanol oxidation to acetic acid is shown in Fig. 5A. Several points can be made regarding fits to this and other models (Fig. 5) as follows. (i) A slow step is required for the conversion of \(E^I\) to \(E^P\) (where \(I\) represents acetaldehyde and \(P\) represents acetic acid). If two rapid C–H bond breaking steps occur in succession and acetaldehyde is not readily released from the enzyme (Table II), no lag in acetic acid formation or appearance would be observed (Fig. 3). (ii) The best fits are seen with rates of chemistry steps (including C–H bond breaking) of ~1000 min⁻¹. These rates are not unreasonable in the context of measured rates of P450 2E1 reduction (10) and the knowledge that the rates measured in the burst experiments were made under conditions of less than saturating substrate conditions (Fig. 4) (10). (iii) \(K_m\) for acetic acid is unimportant because of irreversibility prior to dissociation. Also, there is direct experimental evidence against high affinity (see above). The initial \(K_m\) for simulations was arbitrarily set at 1 M. (iv) The fits for the burst kinetics for ethanol oxidation to acetaldehyde and for acetaldehyde oxidation to acetic acid worked best with \(K_m\) values for ethanol and acetaldehyde set in the millimolar to molar range. These values are not inconsistent with results of binding

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² See Ref. 40 for a general discussion of the meaning of the parameters \(K_m\) and \(V/K\) in enzyme kinetics.
experiments. All of the models shown in Fig. 5, A–C, were able to fit the time course (Fig. 3) and fit the burst results (Figs. 2 and 4) but not the catalytic efficiencies ($k_{cat}/K_m$) or the observed isotope effects.

The best model and set of parameters are presented in Figs. 5D and 6 and Table III, with two distinct forms of the enzyme (E and E') and two forms of the intermediate acetaldehyde, which may be the carbonyl (I) and the gem-diol (Q). A critical feature of the model proved to be the nonenzymatic interconversion of I and Q, assigned as the carbonyl and hydrated forms of acetaldehyde. The model of Fig. 5D could be used with some sets of rate constants that would satisfy all experimental observations, but introducing a slow equilibrium at step 11 was a major problem for matching the observed $K_m$ values. The non-enzymatic reaction is known to have $K_{eq}$ of $\sim 1$, with $k_{11}$ and $k_{-11}$ both $\sim 0.3$ min$^{-1}$ (46). When $k_{11}$ and $k_{-11}$ were set at 1 min$^{-1}$, all experimental constraints could be satisfied when $k_{a1}$ was adjusted to the point that the reaction was irreversible. The model was then very sensitive to the rate used for $k_{a1}$ with the $K_m$ being very sensitive. With $k_a = 6 \times 10^4$ min$^{-1}$, good fits were obtained. This value yielded experimentally determined $k_{cat}/K_m$ and $v(V/K)$ values; higher rates for $k_a$ yielded high values for these two parameters and lower $k_a$ rates yielded low values. The step ($k_a$) is postulated to be the P450 2E1-catalyzed dehydration of the gem-diol of acetaldehyde. In principle, the rate of exchange of acetaldehyde in the presence of the protein could be measured, but the low affinity of acetaldehyde for P450 2E1 and the rate of nonenzymatic exchange make this experiment impractical. Fits of the model and parameters of Fig. 6 to the time course (Fig. 3), ethanol and acetaldehyde burst results (Fig. 4), and isotope effects (Table III) are presented. The $K_m$ values for the oxidation of acetaldehyde to acetic acid may appear to be somewhat low (Table I), but, as pointed out under “Results,” this parameter showed considerable variability. The predicted $v(V/K)$ for this reaction is similar to the value determined experimentally. The predicted $K_d$ values for the two forms of acetaldehyde (Table III) are in the millimolar range. A $K_d$ of 0.3 mM for half of the total acetaldehyde is not inconsistent with the weak inhibition of 4-methylpyrazole binding we observed (see below). Since $K_m = k_{j}/k_{j,k_2}/k_{j,k_3}$ in the conventions of Equation 7, $K_d > K_m$, and the observed $K_m$ value should be $\leq K_d$. Use of the expression $v(V/K) - 1 = K_m/K_d$ from Klinman and Mathews (47) yields a $K_d$ of 0.4 mM for ethanol and 3.5 mM for acetaldehyde. These values are in reasonable agreement with those presented in Table III, considering that the $K_d$ for acetaldehyde (10 mM) and its hydrate (0.3 mM) could both contribute to the estimated $K_d$.

A feature of this model is the use of a separate pathway for the oxidation of acetaldehyde depending upon whether the acetaldehyde is added directly or is formed from ethanol within the enzyme. Possibilities for chemical details are considered in the context of hydrated aldehydes and peroxo-iron chemistry (Fig. 7). Oxidation of ethanol to acetaldehyde probably involves formation of a gem-diol, as shown with the classic hydrogen atom abstraction pathway (Figs. 1 and 7A). Work by Vaz and Coon (48) with rabbit P450 2E1 showed incorporation of label from $^{18}$O$_2$ into benzaldehyde (formed from oxidation of benzyl alcohol). The intrinsic hydrogen isotope effect (10) is also consistent with (but does not prove) the hydrogen abstraction

\[
\begin{align*}
\text{FIG. 2.} & \text{ P450 2E1-catalyzed oxidation of [H]acetaldehyde and [1-2H]acetaldehyde. Reaction mixtures contained 1.0 mM P450 2E1, 3.0 mM NADPH-P450 reductase, 30 mM 1-o-dilauroyl-snglycero-3-phosphocholine, and 2.0 mM cytochrome b$_5$ with 0–1.0 mM [H]-labeled (●) or 0–5.0 mM [H]-labeled acetaldehyde (□). A single set of enzymes was} \\
\text{used in all measurements. The kinetic constants for the [H] substrate} \\
\text{are listed in Table I. With CH$_3$CHO as substrate, $k_{cat} = 50 \pm 0.4$} \\
\text{min$^{-1}$ and $K_m = 1.5 \pm 0.3$ mM ($v(V/K) = 4.5$ and $v(V) = 1.5$). Results are} \\
\text{shown as the average of duplicate rate (○) determinations for each} \\
\text{concentration of substrate. Fits were done using nonlinear regression} \\
\text{analysis (lcplot program) and presented in the form of Hanes-Woolf} \\
\text{plots. Rates are expressed as nmol of acetic acid formed min$^{-1}$ (nmol} \\
\text{of P450 2E1)$^{-1}$.}
\end{align*}
\]

\[
\begin{align*}
\text{FIG. 3.} & \text{ Time course for ethanol oxidation to acetaldehyde (○) and acetic acid (●). The initial ethanol} \\
\text{concentration was 2.0 mM (IP450 2E1) = 1.0 mM). Results are shown as averages of duplicate} \\
\text{experiments. The first time point was 5 s (see also Fig. 4). Lines are} \\
\text{simulated using the models shown in Figs. 5D and 6.}
\end{align*}
\]

\[
\begin{align*}
\text{FIG. 4.} & \text{ Pre-steady-state formation of acetic acid from acetaldehyde. A,} \\
\text{rapid quench kinetic results (inset,} \\
\text{0–1.0 s time points). B, burst analysis for} \\
\text{rapid pre-steady-state phase of product} \\
\text{formation, where } A \text{ represents the burst} \\
\text{amplitude and } k_{a1} \text{ represents the rate of} \\
\text{pre-steady-state burst. Results are shown} \\
\text{as means of duplicate experiments. Inset,} \\
\text{points are from experimental determinations,} \\
\text{and the lines are fits from the models} \\
\text{of Figs. 5D and 6. C, ethanol oxidation} \\
\text{to acetaldehyde (from Ref. 10). [E] = 1.2} \\
\text{μM; [S] = 500 mM. D, acetaldehyde oxidation} \\
\text{to acetic acid (Fig. 4A). [E] = 1.0 μM,} \\
\text{and [I] = [Q] = 1.0 mM.}
\end{align*}
\]
A Minimal Mechanism

\[ \text{E} + S \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} \text{EI} \xrightarrow{k_3} \text{EP} \xrightarrow{k_4} \text{E} + P \]

As written for KINSM:
1. \[ \text{I} \xrightarrow{k_1} \text{E} + I \]
2. \[ \text{E} + S \xrightarrow{k_2} \text{ES} \]
3. \[ \text{ES} \xrightarrow{k_3} \text{EI} \]
4. \[ \text{EI} \xrightarrow{k_4} \text{EP} \]
5. \[ \text{EP} \xrightarrow{k_5} \text{E} + P \]

B Mechanism 2

\[ \text{E} + S \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} \text{EI} \xrightarrow{k_3} \text{EP} \xrightarrow{k_4} \text{E} + P \]

As written for KINSM:
1. \[ \text{I} \xrightarrow{k_1} \text{E} + I \]
2. \[ \text{ES} \xrightarrow{k_2} \text{EI} \]
3. \[ \text{EI} \xrightarrow{k_3} \text{EP} \]
4. \[ \text{EP} \xrightarrow{k_4} \text{E} + P \]

C Mechanism 3

\[ \text{E} + S \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} \text{EI} \xrightarrow{k_3} \text{EP} \xrightarrow{k_4} \text{E} + P \]

As written for KINSM:
1. \[ \text{I} \xrightarrow{k_1} \text{E} + I \]
2. \[ \text{ES} \xrightarrow{k_2} \text{EI} \]
3. \[ \text{EI} \xrightarrow{k_3} \text{EP} \]
4. \[ \text{EP} \xrightarrow{k_4} \text{E} + P \]

D Mechanism 4

\[ \text{E} + S \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} \text{EI} \xrightarrow{k_3} \text{EP} \xrightarrow{k_4} \text{E} + P \]

As written for KINSM:
1. \[ \text{I} \xrightarrow{k_1} \text{E} + I \]
2. \[ \text{ES} \xrightarrow{k_2} \text{EI} \]
3. \[ \text{EI} \xrightarrow{k_3} \text{EP} \]
4. \[ \text{EP} \xrightarrow{k_4} \text{E} + P \]

Fig. 5. Kinetic models for simulation of P450 2E1-catalyzed ethanol oxidation to acetaldehyde and acetic acid. S, ethanol; I, acetaldehyde; P, acetic acid; E, P450 2E1. \(^\text{E}\) represents a conformationally modified form of the enzyme or possibly a chemically distinct substrate, intermediate, or product complex. In the use of the KINSM programs, \( k \) denotes a step governed by an infinitely rapid equilibrium and only controlled by the \( k_{eq} \) = indicates a step governed by both the forward \(( k \) ) and reverse \((- k \) ) rate constants (36). Mechanism 4 (panel D) was utilized in the fits presented later, where \( E \) represents P450 2E1, \( E \) represents a conformationally modified form of P450 2E1 formed during the process of substrate oxidation, \( S \) represents ethanol, \( I \) represents a form of acetaldehyde (postulated to be the aldehyde carbonyl), and \( Q \) represents a form of acetaldehyde different from \( I \), postulated to be the gem-diol. See Fig. 6 for rate constants of individual steps.

### Table III

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Dissociation constants (K\text{eq})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH\text{OH}</td>
<td>0.2 m</td>
</tr>
<tr>
<td>CH\text{CHO} (I)</td>
<td>10 mM</td>
</tr>
<tr>
<td>CH\text{CHOH}_2 (Q)</td>
<td>0.3 m</td>
</tr>
<tr>
<td>CH\text{COH}</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>

#### Parameters for 10-min reactions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( k_{cat} )</th>
<th>( K_m )</th>
<th>( D(V/K) )</th>
<th>( D(V) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol ( \rightarrow ) acetaldehyde</td>
<td>18</td>
<td>20</td>
<td>113</td>
<td>6.2</td>
</tr>
<tr>
<td>Acetaldehyde ( \rightarrow ) acetic acid (^a)</td>
<td>19</td>
<td>0.029</td>
<td>0.082</td>
<td>2.8</td>
</tr>
<tr>
<td>Ethanol ( \rightarrow ) acetic acid (^b)</td>
<td>8.0</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) CH\text{CHOH} or CH\text{CHO}.

\(^b\) CH\text{CHOH}_2 or CH\text{CHOH}.

For \( K \) determinations, the total substrate concentration is the sum of CH\text{CHO} + CH\text{CHOH}_2 and [CH\text{CHO}] = [CH\text{CHOH}].

#### mechanism.

The product could be the gem-diol, which would be detected (as the 2,4-dinitrophenylhydrazone) during analysis. The prospect of iron-catalyzed dehydration of a product gem-diol has been proposed (48), although heme-bound iron is considered a poor Lewis acid (49). If only the aldehyde (carbonyl group) is released, then P450 2E1 would have to catalyze dehydration of the gem-diol to yield a rate consistent with steady-state turnover (Table I).

The form of a carbonyl used as a substrate is a general problem with all enzymes utilizing aldehydes and ketones and is not trivial to answer except under special conditions (50, 51). An explanation for the scheme is that the I and Q forms (Figs. 5D and 6) can be aldehyde carbonyl and gem-diol, respectively. There are possibilities for invoking alternate chemical oxidation mechanisms for the two chemical species, particularly in light of evidence for a peroxo-iron reaction pathway in the P450 oxidation of other aldehydes (Fig. 7) (52, 53). Thus, the postulate can be made that one chemical form (aldehyde or gem-diol)\(^3\) is formed from ethanol in the active site and proceeds directly, while an alternate reaction occurs with added acetaldehyde. Evidence has been presented that P450 19 shows differential kinetic hydrogen isotope effects in the first two steps (methyl hydroxylation, aldehyde formation) of androgen aromatization (43). The \( K_{eq} \) for acetaldehyde and its gem-diol is near unity at neutral pH (46), and both forms of acetaldehyde are available when added to P450 2E1. Another possibility that might be considered is that the gem-triol form of the product carboxylic acid (Fig. 7B) might undergo dehydration as a distinct reaction step. The rate of the nonenzymatic reaction can be calculated at \( 4 \times 10^{-3} \text{ s}^{-1} \) (54), so this is presumed not be be a viable rate-determining step in the aldehyde oxidation reaction.

The \( E \) and \( E' \) forms shown in Figs. 5D and 6 could be the result of changes in protein conformation. Conformational changes have been speculated for other microsomal P450s, including P450s 1A2 and 3A4, the latter of which has been shown to exhibit cooperative behavior (55). Fluorescence and circular dichroism spectroscopy have been used to investigate a potential conformational change for P450 1A2, and results indicate an increase in \( \alpha \)-helical content correlated with enzyme activity (56). Other reports of conformational changes in bacterial and eukaryotic P450s have been published (57, 58); however, there have been no reports suggesting a conformational change for a P450 2E1 enzyme. Conformational changes for prokaryotic P450s have also been revealed through x-ray diffraction analysis. Comparisons between a ferric camphor-bound P450 101 binary complex and a ferrous CO-camphor-bound ternary complex indicate conformational changes in the transition from ferric to ferrous P450 that include repositioning of heme iron in response to movement of the axial Cys ligand and broadening of the CO binding groove (59). Inhibitor-induced conformational changes in P450 101 have been reported (60). Although the protein backbone and heme are essentially

\(^3\) An enol form can also exist but is considered less likely as a substrate for oxidation.
unchanged, dramatic side-chain rearrangement in the sub-
strate binding channel accompanies inhibitor binding. Modeling
studies with P450 102 predict an even more dramatic confor-
national change leading to closure of the substrate ac-
cess channel following substrate binding (58). In the schemes of
Figs. 5D and 6, one form of the enzyme, E, is shown in the start
of an oxidation reaction, and the form $E'$ is shown at the end of
the reaction. A return of $E'$ to $E$ is required for the subsequent
oxidation to begin.

The relevance of these results to other P450 reactions can be
considered. Comparisons with multistep steroid hydroxylases
have already been made (see above). P450 2E1-catalyzed oxida-
sion of urethane is followed by a more efficient epoxida-
tion (62), but these have not been analyzed in detailed pre-
clination studies with P450 102 predict an even more dramatic
conformational change during the course of the oxidation pos-
tulated here will be a general feature of other P450 reactions is
unknown.

Acknowledgments—We acknowledge the contributions of C. G. Turvy
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sion of kinetic simulations.

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312, 59–66
22, 147–159
168–179

In conclusion, we have examined P450 2E1-catalyzed oxidiza-
tion of acetaldehyde to acetic acid and find many kinetic sim-
ilarities to the oxidation of ethanol to acetaldehyde (10). The
significance of the second oxidation step has been overlooked in
previous work with the substrate ethanol. The overall oxidiza-
tion of ethanol to acetic acid is considered as a multistep P450
reaction. Evidence indicates that the intermediate acetalde-
ye does not equilibrate with the medium, and a kinetic model
consistent with experimental results is proposed. This model
involves distinct conformations at the start and end of each
oxidation reaction. Multiple forms of the intermediate aldehyde
are also postulated, plus possibly distinct chemical oxidation
mechanisms. The overall reaction is governed not by affinity of
any of the ligands but by the rates of individual enzyme steps,
with the actual chemical rates of substrate oxidations being
very fast but attenuated by rates of other steps in the catalytic
mechanism. This view of a P450 reaction differs from the con-
cept that selectivity is dictated by static binding interactions
with the substrate in the oxidized protein. Whether or not the
conformational change during the course of the oxidation pos-
tulated here will be a general feature of other P450 reactions is
unknown.

Fig. 6. Values for rate constants in models of oxidation of ethanol and
acetaldehyde by P450 2E1. First-order reaction rates are expressed in units of
min$^{-1}$ and second-order reactions are expressed in units of min$^{-1}$ molar$^{-1}$.

Fig. 7. Possible mechanisms of P450 oxidation of ethanol and
acetaldehyde. A, hydrogen abstraction mechanism for ethanol oxidation. B, hydrogen abstraction mechanism for oxidation of acetaldehyde
gem-diol. C, peroxy-iron mechanism for oxidation of acetaldehyde (car-
bonyl form).
Kinetics of Cytochrome P450 2E1-Catalyzed Oxidation of Ethanol to Acetic Acid via Acetaldehyde

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