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Multi-step oxidations catalyzed by cytochrome P450 enzymes: Processive vs. distributive kinetics and the issue of carbonyl oxidation in chemical mechanisms

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Abstract

Catalysis of sequential oxidation reactions is not unusual in cytochrome P450 (P450) reactions, not only in steroid metabolism but also with many xenobiotics. One issue is how processive/distributive these reactions are, i.e. how much do the “intermediate” products dissociate. Our work with human P450s 2E1, 2A6, and 19A1 on this subject has revealed a mixture of systems, surprisingly with a more distributive mechanism with an endogenous substrate (P450 19A1) than for some xenobiotics (P450s 2E1, 2A6). One aspect of this research involves carbonyl intermediates, and the choice of catalytic mechanism is linked to the hydration state of the aldehyde. The non-enzymatic rates of hydration and dehydration of carbonyls are not rapid and whether P450s catalyze the reversible hydration is unknown. If carbonyl hydration and dehydration are slow, the mechanism may be set by the carbonyl hydration status.

Keywords

Cytochrome P450; catalytic mechanisms; kinetics; carbonyl oxidation; aromatase; nitrosamines

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Highlights

- Cytochromes 2A6 and 3E1 catalyze sequential oxidations of xenobiotic chemicals with processive kinetics.
- Cytochrome P450 19A1 catalyzes the 3-step aromatization of androgens to estrogens in a distributive manner.
- At least three chemical mechanisms can be proposed for the oxidation of an aldehyde to a carboxylic acid by cytochrome P450.
- The potential mechanisms of aldehyde oxidation are dependent upon the state of hydration of the carbonyl, which is a slow non-enzymatic process.

Introduction

P450¹ enzymes, as a group, catalyze oxidations of what are probably the most diverse set of substrates involved with any enzyme system [1]. These enzymes, however, do use a rather limited chemical reaction to achieve these oxidations [2,3], with much of the pageantry of the reaction repertoire resulting from rearrangements, either at the level of reaction intermediates or unstable products. Understanding the details of the chemistry of catalysis in P450 reactions has been demonstrated to be extremely important in the drug development process in the pharmaceutical industry, in terms of (i) understanding what were regarded as highly unusual biotransformation processes [3] and (ii) improving predictions about the transformation of drugs and drug candidates to toxic products [4].

Most studies on P450 catalysis have been focused on single oxidations, in that these are the simplest. However, a number of steroid oxidations are actually 3-step processes, i.e. they consist of sequential 2-electron oxidations (e.g. human P450s 11A1, 11B2, 17A1, 19A1, and 51A1 plus numerous reactions in other species) (Fig. 1) [2,5]. A key question is how processive these multi-step reactions are, i.e. the extent to which the oxidation product of one step stays on the enzyme, without dissociating, before the next step. An example of the relevance is seen with P450 19A1, the steroid aromatase and an important target in treating breast cancer. If an intermediate product does not dissociate from the enzyme, then developing an inhibitor based on that structure might be unproductive.

Background

Of the 57 human P450s, at least five catalyze important multi-step steroid oxidations (Fig. 1). The processivity of some of these reactions (i.e. 11B2, 17A1) has been studied, mainly by Kominami's group [6-8]. The following conclusions have been proposed, based mainly upon pulse-chase experiments. P450 11B2 has been reported to convert deoxycorticosterone to aldosterone without the dissociation of intermediates from the enzyme, on the basis of pulse chase and quench experiments [8]. With P450 17A1 about 20% of the intermediate 17 α -hydroxypregnenolone did not dissociate from the enzyme in the oxidation of pregnenolone to dehydroepiandrosterone [7], although a study with P450 17A1 transfected into HEK-293 cells yielded a different conclusion [9]. Prior literature with P450 19A1, the steroid aromatase, is controversial regarding processivity [10]. Rat P450 2C11, a P450 which does not have a defined physiological role in steroid metabolism, oxidizes testosterone to 16 α -hydroxyandrostenedione via androstenedione, and Sugiyama *et al.* [11] estimated that ~15% of the androstenedione intermediate does not dissociate in the process. To our knowledge, similar studies have not been reported with P450s 19A1 and 51A1.

In addition to these steroids (Fig. 1), the literature contains a number of reports of non-mammalian P450 systems that catalyze such multi-step reactions and also mammalian P450s that catalyze multi-step oxidations of xenobiotics. Of course, most drugs undergo multiple processing steps in the body, but in many cases multiple enzymes (and multiple P450s) are involved so the compounds necessarily dissociate and become substrates for other enzymes. A well-known example is the sequential oxidation of the carcinogen benzo[*a*]pyrene to an epoxide and then to a diol epoxide (P450s 1A1 and 1B1 both do this [12]) but the sequence is interrupted with the action of epoxide hydrolase. This list of reactions catalyzed by a single P450 (not intended to be comprehensive) includes P450 1A2-catalyzed oxidation of pyrene to 1-hydroxypyrene and then to several dihydroxy pyrenes [13]. Human P450 1A2 also bioactivates a pyrazolopyrimidine compound by hydroxylation followed by further oxidation to what can be considered a vinylogous iminoquinone [14]. Another example is the desaturation of ethyl carbamate (urethane) by P450 2E1 followed by the more efficient epoxidation of the intermediate vinyl compound [15]. In numerous cases the ω -oxidation of a

fatty acid is catalyzed by a P450 and the product goes on to yield a dicarboxylic acid, although alcohol and aldehyde dehydrogenases may catalyze the latter steps instead of P450s.

P450 2E1

The original goal of the study with P450 was to understand the basis of the kinetic hydrogen isotope effect on the K_m (but not k_{cat}) for the *N*-demethylation of DMN [16], which had been observed in rat liver microsomes following the report of a strong kinetic deuterium isotope on the carcinogenicity of this compound in rats [17]. Kinetic analysis of the oxidation of ethanol to acetaldehyde (Fig. 2), a classic reaction of P450 2E1 [18,19], revealed burst kinetics for the reaction with a rate of $\sim 6 \text{ s}^{-1}$ followed by a steady-state turnover of 0.3 s^{-1} (Fig. 3) [20]. The kinetic deuterium isotope effect was expressed in the burst phase. Therefore the rate-limiting step (in the steady-state) follows product formation and does not show the isotope effect. The expression for K_m is complex and does include the isotope effect [20].

P450 2E1 also oxidizes acetaldehyde to acetic acid [22,23], and studies with human P450 2E1 showed that most of the product produced from ethanol in 10 min was acetic acid [21]. The k_{cat} and K_m for the second reaction were not particularly favorable, but pulse chase experiments (with radioactive ethanol followed by the addition of carrier acetaldehyde) revealed that most of the acetic acid was formed without exchange of the acetaldehyde from P450 2E1. The results are not due to any inherent high affinity of P450 2E1 for acetaldehyde [21].

A kinetic model was developed that can explain all of the experimental data (Fig. 4). One issue, to be discussed later, is the relatively slow hydration of aldehydes (and also dehydration of hydrated aldehydes, i.e. *gem*-diols). The model (Fig. 4), constructed using the program KINSIM [20], is based on the knowledge that P450s undergo conformational changes upon binding substrates, as seen with several crystal structures [24]. If a P450 undergoes a conformation change upon binding substrate, then it must also revert to the original conformational state after the reaction, either before or after release of the product. Thus, a model was constructed in which a slow conformation change step was included after product formation, and the non-enzymatic rates of acetaldehyde hydration (and dehydration of the *gem*-diol) were included (Fig. 2) [21]. Thus, the kinetic features of this reaction are quite different than those proposed for most P450s, with rapid oxygen activation and oxygenation followed by rate-limiting putative conformational change following product formation. It should be pointed out that another (one-step) P450 2E1 reaction, lauric acid 11-hydroxylation, did not show burst kinetics [20].

P450 2A6

As mentioned earlier, an original impetus for the work with P450 2E1 was the observed kinetic deuterium isotope effects for dialkyl nitrosamine oxidation and carcinogenicity [16,17,25]. DMN and DEN were synthesized with a variety of deuterium labels and used as substrates with human P450 2A6, which is known to oxidize both DMN and DEN (Fig. 5), (the latter at a faster rate) [26,27]. The oxidations showed some, but not all, of the characteristics of the P450 2E1 reaction with ethanol [28]. A high kinetic deuterium isotope effect was observed for the oxidation of DMN to formaldehyde, but only a modest isotope effect was found for oxidation of DEN to acetaldehyde. Only partial kinetic bursts were seen, and the high isotope effect for DMN oxidation was expressed in both k_{cat} and K_m .

As in the case with P450 2E1 oxidation of ethanol, a substantial fraction of the aldehyde (formaldehyde in the case of DMN and acetaldehyde in the case of DEN) was converted to

the carboxylic acid [28]. Kinetic modeling based on the k_{cat} and K_{m} values for the individual reaction steps predicted a lag in the formation of the carboxylic acids, but no lag was seen and the model underestimated the production of carboxylic acid. Pulse chase experiments (with labeled nitrosamines) indicated that only 10-60% of the aldehyde dissociated from P450 2A6 in the overall oxidation of the nitrosamines to the carboxylic acids (Fig. 6) [28].

Kinetic models were developed in which P450 2A6 undergoes a conformational change upon binding the nitrosamine, forms the aldehyde product, and is then in a conformation to oxidize the aldehyde to carboxylic acid, without dissociation of the aldehyde (Figs. 7, 8) [28]. As with P450 2E1, this processivity cannot be attributed to an inherent high affinity of P450 2A6 for the aldehyde.

The kinetic model [28] can explain the observed results but another mechanism is possible (Fig. 9). The α -hydroxy nitrosamine has a known half-life and, in principle, could be oxidized to a nitrosamide. The latter compound was synthesized [29] and had a $t_{1/2}$ of 50 s under the conditions used (pH 7.4, 37 °C). This compound had been synthesized previously and reported to be a direct-acting mutagen [30]. The analysis of the α -hydroxy nitrosamine (generated by hydrolysis of the acetate ester [31]) and the nitrosamide can be done by LC-UV, although the short half-lives of the compounds raise the limits of sensitivity. Attempts with P450 2A6 incubation of DMN or the acetoxy ester of α -hydroxy DMN have been unsuccessful in demonstrating the formation of the nitrosamide, at least to date.

P450 19A1

P450 19A1 is the aromatase that converts androgens to estrogens (Fig. 1). This enzyme plays a critical role in steroid metabolism, and genetic deficiencies have serious consequences [32]. This enzyme is also a therapeutic target in estrogen-positive breast cancers, and several inhibitors have been developed as drugs [33]. Thus, understanding the catalytic mechanism and processivity of this enzyme is important, e.g. designing an inhibitor based on an intermediate might not be helpful if the reaction is highly processive.

Kinetic analysis was done with *Escherichia coli* recombinant human P450 19A1, with the heterologous expression and purification described in detail [34]. Androstenedione, its 19-hydroxy and 19-aldehyde products, and the final product estrone were all found to bind to P450 19A1 and produce a partial low- to high-spin iron shift. In each case the binding kinetics of the spin shift were slow (following rapid mixing) and were best fit to biphasic plots [34]. The slow rate of the spin shift is proposed to be due to the movement of each ligand into the active site, not the initial interaction with the P450, because a rapid quench of fluorescence was observed upon mixing the fluorescent inhibitor α -naphthoflavone [35] with P450 19A1. This behavior is similar to that previously reported for P450 3A4 [36].

Androstenedione and its 19-hydroxy and 19-aldehyde products are all converted to estrone, and k_{cat} and K_{m} parameters were measured for each transformation. Only in the latter case is a single oxidation step involved.

A key finding is the lack of processivity observed in pulse chase experiments with radiolabeled androstenedione and carrier 19-hydroxy and 19-aldoandrostenedione; i.e. no excess label was carried through to estrone (Fig. 10) [34]. A single-turnover reaction initiated with P450 19A1 and a single equivalent of androstenedione yielded a kinetic trace in which the 19-hydroxy and 19-aldehyde products were formed and oxidized sequentially. The data points could be fit to a distributive mechanism with rate constants that are consistent with several other parts of the data for P450 19A1 (Fig. 11) [34].

The results are somewhat surprising, in that one might intuitively expect a “physiological” reaction such as androgen conversion to an estrogen to be more efficient if processive than distributive. However, P450 19A1 androstenedione oxidation is distributive [34] and the oxidation of the xenobiotic dialkyl nitrosamines by P450 2A6, a non-essential P450 [37], is processive [28].

The Aldehyde Hydration Dilemma

In considering P450s 2E1, 2A6, and 19A1, a common element is the formation of an aldehyde, prior to the last oxidation to a carboxylic acid (Figs. 1, 2, 5). Closer examination of the multi-step steroid oxidations in Fig. 1 also shows aldehyde or ketone intermediates in all but one case (P450 11A1).

Aldehydes exist as mixtures with their hydrates, with the equilibrium depending upon the substituents of the aldehyde carbon. For instance, acetaldehyde exists as a ~ 1:1 mixture with its hydrate in an aqueous solution at neutral pH [38,39]. The rates of hydration and dehydration have been measured in some cases and are relatively slow (Fig. 7) [40-42].

The dilemma is whether the aldehyde or its hydrate is the substrate (Fig. 12). The two-step oxidation of ethanol with a generalized hydrogen atom abstraction/rebound mechanism yields a *gem*-triol (Fig. 2B), which can dehydrate to the carboxylic acid. The carbonyl hydrogen atom could alternatively be abstracted directly (followed by an oxygen rebound) (Fig. 2C). The C-H bond strength is weak enough for hydrogen abstraction, which is documented in lipid peroxidation chemistry and other fields [43,44], including previous proposals for P450 [45,46]. Alternatively the unhydrated aldehyde can be oxidized using a ferric peroxide mechanism (Fig. 2D), which was originally developed to rationalize observations made with the P450 19A1 reaction [43-46]. In particular, the report that ^{18}O label derived from $^{18}\text{O}_2$ in the product formic acid [43] provides what appears to be the strongest evidence supporting the peroxide mechanism.

With the P450 2E1 oxidation of ethanol (Fig. 2), the rates of acetaldehyde hydration and dehydration are known, at least in the uncatalyzed reactions (Fig. 7) [40,41]. The oxidation of ethanol would be expected to produce a *gem*-diol, which could undergo a subsequent hydrogen abstraction followed by oxygen rebound to yield the *gem*-triol, so an actual aldehyde does not need to be inferred.

Using the mechanism of Fig. 7 for the oxidation of DMN or DEN, the decomposition of the α -hydroxy nitrosamine yields an aldehyde (not hydrated). If the “conventional” hydrogen abstraction/oxygen rebound mechanism is involved, then the aldehyde may or may not be hydrated. Hydration is a relatively slow reaction (0.005 s^{-1}) [40,41] and not compatible with the overall rate of oxidation of the dialkyl nitrosamine to carboxylic acid. If hydration is slow, then it is necessary to invoke a ferric peroxide mechanism or the direct abstraction of the carbonyl hydrogen atom for the oxidation of the aldehyde.

In the second step of the P450 19A1 reaction, oxidation of 19-hydroxyandrostenedione leaves the C-19 group as the *gem*-diol. A mechanism has been proposed in which this is used as the substrate in the third step of the reaction, with a hydrogen abstraction from C-2 [51]. That mechanism does not account for the report that ^{18}O from $^{18}\text{O}_2$ is present in the desired formic acid [47]. However, if the proposed ferric hydroperoxide mechanism [47] is operative, then the C-19 *gem*-diol must dehydrate first (Fig. 13). A mechanism involving abstraction of a hydrogen atom from the aldehyde is probably not tenable in that it would be expected to result in an androgen with a carboxylic acid at the C-19 position, not an estrogen.

These three P450 reactions (P450s 2E1, 2A6, 19A1) present the dilemma. In none of these cases is it known with certainty whether the hydrogen atom/oxygen rebound or ferric hydroperoxide mechanism is preferred. The P450 2E1- [21] and P450 2A6- [28] catalyzed oxidations of acetaldehyde both show non-competitive intermolecular kinetic deuterium isotope effects of ~ 2 (on k_{cat}/K_m). In principle, the ferric hydroperoxide mechanism might not show a kinetic deuterium isotope effect, in that a C-H bond is not being broken until later in the reaction (Fig. 2D). However, it is not presently possible to compare the relatively low kinetic isotope effects [21,28] to intrinsic isotope effects to determine the extent of expression (of the isotope effect) in these reactions.

Another problem in these considerations of mechanisms is the limited knowledge of rates of hydration and dehydration, beyond the simple short-chain aldehydes [39-41]. The equilibrium between an aldehyde and its hydrate can be readily analyzed by the ^1H NMR spectrum (in D_2O), in that the proton of the *gem*-diol is considerably upfield of that of the aldehyde. Rates of exchange can be established using ^{17}O NMR, either by direct observation of the ^{17}O signal [41] or indirectly by ^1H - ^{17}O coupling. Another option, within a reasonable time scale, is the use of mass spectrometry to measure rates of exchange from H_2^{18}O (with an observed rate being the sum of the forward and reverse rate constants). Another complication is that it is currently unknown whether P450s catalyze the rates of hydration of carbonyls (or dehydration). In principle, rates of H_2^{18}O exchange can be used to address this question, although rapid reactions are probably beyond the scope of measurement unless a method such as rapid reaction kinetic mass spectrometry [52] is applied.

Other considerations

The discussions have been focused largely upon reactions in which alcohols are oxidized to aldehydes and then to carboxylic acids (e.g., P450s 2E1 and 19A1). However, the P450 2A6 dialkyl nitrosamine study [28] is a variant of a possibly more general situation, *N*-dealkylation of an amine followed by oxidation of the resulting aldehyde. The “2-step” character of these reactions has not been explored in as much depth, but some possible examples are any *N*-demethylation or *N*-deethylation catalyzed by P450s 2A6 or 2E1, in that the oxidations of formic and acetic acid by these P450s have been characterized [21,28].

In these situations, a feature is the rate of cleavage of the carbinolamine (followed by the same dilemma of whether the resulting aldehyde will be hydrated for further oxidation). The literature contains a number of examples of oxidations of cyclic amines to lactams, e.g. nicotine, 3-(*p*-chlorophenyl)pyrroline, and *N*-methyl-4-phenyl-1,2,5,6-tetrahydropyridine [53]. Presumably the lactams result from the oxidation of stable carbinolamine intermediates. The P450-catalyzed formation of amides (from amines) has less precedent, if any, in acyclic compounds but should be possible. The literature contains a number of kinetic studies regarding carbinolamines, although much is devoted to the rates of dehydration (to imines) [54-57] as opposed to the release of the amine and carbonyl. As in the case of the issue of hydration and dehydration, there is no information about how much the enzyme (P450) might facilitate this reaction through acid-base chemistry.

Conclusions

A number of multi-step P450 reactions are known, including both endobiotic and xenobiotic substrates. The available evidence indicates that the degree of processivity varies considerably and is not predictable in the absence of detailed experiments. An important issue in many of the multi-step reactions is a carbonyl intermediate and its hydration state. The uncatalyzed rates of hydration of carbonyls (and dehydration) are relatively slow. Unless P450s are found to accelerate the addition of water to carbonyls and the dehydration of *gem*-diols, the P450s may be committed to either hydrogen atom abstraction or ferric

peroxide mechanisms (Fig. 2). If a particular P450 is found to catalyze hydration and dehydration, it is unlikely that the result can be widely extrapolated among other P450s.

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Abbreviations

DEN	<i>N,N</i> -diethylnitrosamine (<i>N</i> -nitrosodiethylamine)
DMN	<i>N,N</i> -dimethylnitrosamine (<i>N</i> -nitrosodimethylamine)
P450	cytochrome P450

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Fig. 1.
 Multi-step oxidations of steroids catalyzed by human P450 enzymes [5].

Fig. 2. Stepwise oxidation of ethanol to acetic acid by human P450 2E1. Three possible mechanisms are shown for the oxidation of acetaldehyde to acetic acid in parts B-D.

Fig. 3. Pre-steady-state kinetic burst observed for oxidation of ethanol to acetaldehyde with P450 2E1 [20].

Fig. 4.

A kinetic mechanism for the oxidation of ethanol to acetic acid by P450 2E1 [21]. Rate constants are shown in units of s^{-1} and M^{-1} .

Fig. 5.
Oxidation of DMN and DEN to aldehydes and carboxylic acids by P450 2A6.

Fig. 6.

Pulse chase experiments with P450 2A6 [28]. Reactions were initiated with labeled nitrosamines. After the indicated time, unlabeled aldehyde (1.5 or 2 mM) was added and the reactions proceeded 20 min, at which time the labeled carboxylic acids were quantified as described in the original reference. (A) DMN. (B) DEN.

Fig. 7.

A general scheme of oxidation of dialkyl nitrosamines [28]. The indicated rate constants are for non-enzymatic hydration and dehydration of the aldehydes [40,41] and for hydrolysis of the α -hydroxy nitrosamine [42].

Fig. 8.
A kinetic mechanism for oxidation of dialkyl nitrosamines by human P450 2A6 [28].

Fig. 9.

An alternate mechanism for the oxidation of alkylnitrosamines to carboxylic acids, with the intermediate α -hydroxy nitrosamine serving as a substrate for the second reaction. The half-lives of the unstable products are indicated (at pH 7.4, 37 °C)

Fig. 10.

Pulse chase experiments with human P450 19A1. The reaction was initiated with [^{14}C]androstenedione, and the indicated compounds were added after 1 min, followed by further incubation for 19 min and analysis of radiolabeled estrone [34].

Fig. 11.
Fitting of a single-turnover P450 19A1 androstenedione reaction using the model and rate constants of reference [34] to a minimal kinetic mechanism.

Fig. 12.
Alternate chemical mechanisms for the third step of the P450 19A1 oxidation.

Fig. 13. The hydration issue with the aldehyde in the third step of the P450 19A1 reaction, which is general for multi-step P450 oxidations involving carbonyl intermediates.