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Rapid kinetic methods to dissect steroidogenic cytochrome P450 reaction mechanisms

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Abstract

All cytochrome P450 enzyme reactions involve a catalytic cycle with several discrete physical or chemical steps. This cycle ends with the formation of the reactive heme iron-oxygen complex, which oxygenates substrate. While the steps might be very similar for each P450 enzyme, the rates of each step varies tremendously for each enzyme and sometimes even for different reactions catalyzed by the same enzyme. For example, the rate-limiting step for most bacterial P450 enzymes, with turnover numbers over 1,000 sec⁻¹, is the second electron transfer. In contrast, steroidogenic P450s from eukaryotes catalyze much slower reactions, with turnover numbers of ~5–250 min⁻¹; therefore, assumptions about kinetic properties for the mammalian P450 enzymes based on the bacterial enzymes are tenuous. In order to dissect the rates for individual steps, special techniques that isolate individual steps and/or single turnovers are required. This article will review the theoretical principles and practical considerations for several of these techniques, with illustrative published examples. The reader should gain an appreciation for the appropriate methods used to interrogate particular steps in the P450 reaction cycle.

Graphical abstract

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1. Cytochrome P450 enzymes

Omura and Sato first discovered the P450 enzymes from rabbit liver microsomes in 1962 [1]. Human beings have 57 P450 enzymes, which catalyze a variety of reactions using a broad range of substrates. The products of these reactions include steroid hormones, active vitamins and other bioactive lipids, and xenobiotic metabolites. Despite the diversity of P450 chemistries, most reactions are variations of the basic oxygen insertion reaction in a C-H bond, equivalent to the hydroxylation of alkane substrates. The catalytic cycle for this process was first elucidated through the study of bacterial enzymes such as P450cam (P450 101), which led to many of the fundamental concepts in the field. Over time, individual steps were thoroughly studied, by trapping intermediates for spectroscopic characterization.

Following the cloning of cDNAs encoding eukaryotic P450s and the advent of methods to express these enzymes in bacteria, these eukaryotic enzymes have been studied in reconstituted systems with purified proteins. The hepatic P450s have attracted considerable attention, due to their central importance in drug metabolism. The steroidogenic P450s have also been the focus of intense study, due to their prominence in health and disease. The human steroidogenic enzymes include the 3 mitochondrial enzymes CYP11A1 (P450 11A1, P450_{scc}, cholesterol side chain cleavage enzyme), CYP11B1 (P450 11B1, P450_{c11β}, steroid 11β-hydroxylase), and CYP11B2 (P450 11B2, P450_{c11AS}, aldosterone synthase), as well as the 3 microsomal enzymes CYP17A1 (P450 17A1, P450_{c17}, steroid 17α-hydroxylase/17,20-lyase), CYP21A2 (P450 21A2, P450_{c21}, steroid 21-hydroxylase), and CYP19A1 (P450 19A1, P450_{aro}, aromatase). Mutations in the genes encoding the steroidogenic P450 enzymes are all associated with human diseases, and 4 of these enzymes are targets of drugs [2]. Consequently, a detailed understanding of their structures and catalytic mechanisms are important to conceptualize disease-causing mutations and to develop drugs selective for a specific enzyme.

2. Steady-State Enzyme Kinetics Versus Rapid Kinetics

Conventional enzyme kinetic studies determine values for the familiar Michaelis constant (K_m) and maximal rate (V_{max}) or turnover number (k_{cat}). These experiments involve steady-

state measurements of many turnovers for each enzyme molecule over many seconds to minutes. For a P450-catalyzed reaction, each enzyme molecule completes multiple cycles in these steady-state assays, and the rate of product formation reflects the rate of the entire cycle, with no information gleaned about the rate of individual steps. As a general principle of enzymology, however, the overall rate of the reaction is governed by the rate of slowest step in the process, or the rate-limiting step in the sequence. A step is partially rate-limiting if its rate is within a factor of ~5 of other steps, according to the equation:

$1/k_{ss} = \sum_{(i=1)}^n 1/k_i$, where k_{ss} is the overall reaction rate at steady-state and k_i represents the rates of $n=i$ individual steps. With advances in enzymology techniques such as stopped flow, rapid quench, and isotope labeling studies, each individual step of the P450 catalytic cycle can be studied in detail for wild type and mutant variants. These techniques may be employed to determine rates generally on the millisecond timescale, which is typical for most P450 reactions [3].

2.1. Individual Steps in the P450 Catalytic Cycle (9 Different Steps)

Individual steps in the P450 catalytic cycle for microsomal enzymes include (Scheme 1): 1. substrate binding, 2. first one-electron reduction by P450-oxidoreductase (POR), 3. oxygen binding, 4. second one-electron reduction by POR, 5. protonation of the distal oxygen coordinating to iron, 6. formation of the reactive heme iron-oxygen species (compound I), 7. hydrogen atom abstraction, 8. oxygen rebound with the radical intermediate, 9. product release. For mitochondrial P450 enzymes, the cycle is the same, except the electron donor is the iron-sulfur protein ferredoxin (FDX1, or adrenodoxin), which acts as an electron carrier between the P450 enzyme and ferredoxin reductase (or adrenodoxin reductase) [4]. The experimentally measurable steps discussed in this review are steps 1, 2, 7, and 9. Steps 1, 2, and 9 (i.e. substrate binding, electron transfer, and product release) will be discussed in Section 3.1, where we will describe stopped-flow and binding titration techniques. Section 3.2.1 will also discuss step 9, which will define the experimental setup for rapid chemical quench techniques. Section 4 will discuss the use of kinetic isotope effects to measure step 7 (i.e. C-H abstraction). Although not discussed in detail in this manuscript, there have been reports of measuring the rate of oxygen rebound (step 8) using radical clock substrates [5–7]. These cyclopropane-containing substrates yielded oxygen rebound rates (step 8) in the nanosecond time scale ($2\text{--}20\text{ ns}^{-1}$) [5].

2.2. Spectroscopic Measurement of Substrate, Product, or Inhibitor Binding

The heme prosthetic group of P450 enzymes exhibits an absorption spectrum in the 400–500 nm (UV-Vis) range, featuring a red-shifted Soret band ($\pi \rightarrow \pi^*$ transition) with a peak at 385–425 nm. This spectrum is very sensitive to changes in ligands and redox state and hence changes with each step in the P450 reaction cycle. Consequently, progress along the P450 cycle can be monitored by UV-Vis, at least for intermediates with a lifetime long enough to detect spectroscopically. In addition, other ligands that shift the Soret band might be employed to trap intermediates. For example, carbon monoxide (CO) is a high-affinity ligand for binding to the iron in the ferrous (Fe^{+2}) oxidation state, which is explained by π -backbonding of the unoccupied π^* orbital of CO interacting with the occupied d-orbital of iron (Dewar-Chatt-Duncanson model) [8]. The rapid binding of CO to reduced enzyme

shifts the Soret band to 450 nm, which is the origin of the “P450” name for these enzymes (Fig. 1). The 450 nm absorption is used to determine the concentration of purified P450 enzymes and to infer the rate of the first electron transfer step. To increase the sensitivity and to simplify the spectra, difference spectra are often obtained, subtracting the spectrum of enzyme in state A in the reference cuvette from an equal concentration of enzyme in state B in the sample cuvette. In addition to ligands, inorganic reductants such as sodium dithionite can be used to supply the first (but not the second) electron and to artificially move the enzyme along in the cycle without POR or FDX1.

In addition to redox state and CO ligand, substrate and inhibitor binding also induce characteristic spectral changes of the Soret band. The binding of a substrate that also displaces the axial water ligand (Scheme 1, state **A** to **B**) induces a change in the iron atom from a low-spin to a high-spin state [9]. In the presence of the substrate, the absorption maximum of the Soret band shifts to a shorter wavelength from spectrum [*iv*] at 418 nm (Fig. 2A) to spectrum [*i*] at 394 nm – a blue shift. A corresponding *type I difference spectrum* (Fig. 2B), subtracting spectrum [*iv*] from spectrum [*i*], shows a maximum or “peak” at 390 nm and a minimum or “trough” at 420 nm [10, 11]. When nitrogen-containing inhibitors—typically azoles and related heterocyclic moieties—bind the enzyme and form a complex containing a strong Fe-N bond, the maximum of the Soret band shifts to longer wavelength, from 418 nm to 425 nm – a red shift (Fig. 2A, spectrum [*ii*]) [12]. A corresponding difference spectrum has a minimum at 390 nm and maximum at 425 nm, which is called a *type II difference spectrum*, subtracting spectrum [*iv*] from spectrum [*ii*] (Fig. 2C). Some ligands induce a reverse-type I binding spectrum, which is explained by a population of high-spin enzyme in the resting state and induction of low-spin state with water or substrate hydroxyl group liganding the heme iron [13]. Based on observations with electron paramagnetic resonance (EPR) spectroscopy [14] and UV-Vis absorption spectra, the high-spin state corresponded to type I ligands [15] and the low-spin state was related to type II ligands [16]. The energy diagram of the d-orbitals for a low-spin ferric (Fe^{+3}) complex is considered in Figure 3 – the paramagnetic character allows for sensitive measurement of iron complexes by EPR spectroscopy [17].

The spectral changes that occur upon enzyme binding to substrates or inhibitors allow one to measure K_d (or the spectral binding constant, K_s) of the substrate or inhibitor. The K_d is determined by plotting the peak-to-trough value from the difference spectra with and without inhibitor as a function of ligand concentrations to determine the concentration where the change is half-maximal by curve fitting. Although nitrogen-containing compounds tend to give type II binding changes, not all aza-aromatic compounds yield a type II binding mode, and more importantly, they do not always lead to inhibition of the enzyme. For instance, there have been cases where P450 enzymes metabolize compounds that induce a type II binding spectrum. An insightful study showed that type II substrates had a higher *in vitro* metabolic clearance by the enzyme (i.e. catalytic efficiency V/K) compared to the type I counterparts [19]. The authors explain that their type II ligands had higher affinity, which increased the metabolic clearance.

3. Stopped-Flow Technique (Steps 1, 2, 9 – Substrate Binding, First Electron Reduction, Product Release)

The enzymologist can take advantage of these spectral changes upon ligand binding to design experiments that will measure the rates of certain individual steps (see section 3.1).

3.1. Stopped-Flow with Spectroscopy

A stopped-flow apparatus may be used to measure the binding rate of the substrate to the enzyme. Figure 4 illustrates the approach used to determine the kinetics of testosterone binding to aromatase (CYP19A1) [22]. By analogy, the rate of change in the UV-Vis spectrum yields individual k_{on} and k_{off} rates for the ligand. These spectral changes are typically too rapid to measure in a conventional spectrophotometer and require the use of a stopped-flow instrument for precise sampling on the millisecond time scale. Stopped-flow instruments enable rapid mixing of enzyme and reagents with prompt and continuous measurement of some parameter such as UV-Vis absorbance. In this experiment, the stopped-flow instrument would have two syringes—one syringe containing the enzyme and the other syringe containing the ligand—and the mixing chamber would immediately deliver the solution to a chamber equipped with a spectrophotometer to monitor the shift in the Soret peak as a function of time. If the different ligand concentrations yield different rates of binding, then a plot of k_{obs} vs. $[S]$ yields a linear equation, which is fit to a 2-state model: $k_{\text{obs}} = k_{\text{on}}[S] + k_{\text{off}}$, where k_{on} is the slope and k_{off} is the y-intercept, while k_{obs} is the measured rate of spectral change. The k_{off} rate of a substrate is more difficult to measure but can be determined if a rapid- and tight-binding inhibitor such as ketoconazole is available. In this case, the first syringe would contain ligand bound to enzyme, and the second syringe would contain a large excess of inhibitor. The spectrophotometer would monitor the change from type I to type II spectrum.

Variations in the stopped flow-method can be used to interrogate other steps in the cycle. For example, one could measure the rate of the first electron transfer by mixing a CO-saturated solution of enzyme in one syringe with POR and NADPH in the other syringe and monitoring the change in absorbance at 450 nm. As soon as electron transfer occurs, CO will bind the ferrous iron (Fe^{+2}) and shift the Soret band [23]. An example of this experimental setup was nicely illustrated with CYP11B1 and its electron-transfer partners, FDXR and FDX1. One syringe contained the enzyme, CYP11B1, and the other syringe contained NADPH, FDXR and FDX1, while both syringes were saturated with CO [24]. Upon rapid mixing, the iron in CYP11B1 was reduced from Fe^{+3} to Fe^{+2} and immediately formed the CO complex with spectral shift to 450 nm. The rate of the spectral change reported the rate of CYP11B1 reduction and yielded three phases, with rate constants of 60 s^{-1} , 3.9 s^{-1} , and 0.65 s^{-1} , suggesting the presence of different conformational species between redox partners and enzyme.

3.2. Rapid Quench Technique

3.2.1. Rapid Chemical Quench (Step 9, Product Release)—Rapid chemical quench is another technique to study the product release step (Figure 5). In the special case where product release is rate-limiting, a distinguishing feature of the reaction is that the

formation of the first product molecule for each enzyme molecule is faster than subsequent turnovers, which do not occur until product is released. For these systems, a burst phase occurs, because the initial velocity of the reaction during the first turnover is faster than subsequent turnovers, and once all of the enzyme molecules have formed product, the rate reflects the steady-state rate. A rapid chemical quench apparatus can be used to measure burst phase kinetics in an enzyme that catalyzes a single chemical step with a rate k_2 (Figure 6) by taking multiple samples during the time required for all enzyme molecules to form one molecule of product. An exhaustive list of spectroscopic techniques will not be discussed in this section, but another technique worth mentioning is resonance Raman spectroscopy, which selectively measures the vibrational modes of the heme group in P450 enzymes. This technique was used to observe the different P450 species depending on the substrate used [25, 26].

In Figure 6A, the ES complex could be a series of intermediates (ES1, ES2, ES3, etc) such as the intermediates **B**, **C**, **D**, **E**, **F**, and **G** in the P450 catalytic cycle (Scheme 1), while the EP is the nascent enzyme-bound product complex (intermediate **I** in Scheme 1).

This experimental set up has been employed in the study of CYP21A2 [27, 28]. The design is to use an excess of substrate (1 μ M enzyme in one syringe and 20 μ M substrate in the other) in the rapid-quench apparatus. A series of experiments are performed pushing substrate and enzyme at a constant flow rate between the point of mixing and the quench chamber, containing acid or other reagent to stop the reaction. By varying the length of the tubing (loop) connecting the mixing point and the quench chamber, the time of the reaction is varied. Samples are obtained on the millisecond time scale at several times prior to the formation of one molar equivalent of product to determine the initial rate of the reaction. In the case of 17-hydroxyprogesterone, no burst was observed; however, when progesterone was used as the substrate, a burst was observed [28]. Thus, the rate-limiting step can be substrate-specific for a given P450.

Although a rate-limiting product release step can explain the observation of a burst (Fig. 6A, k_3), it may not necessarily be the only explanation for the burst. For example, the reversible equilibrium of an enzyme-substrate complex between an active form and an alternate form can explain a partial burst. For example, the observation that the substrate progesterone (**1**) yields both 11-deoxycorticosterone (**2**) and 16 α -hydroxyprogesterone (**3**) with CYP21A2 suggests that a conformer of the ES complex (Fig. 7, ES') exists so that the 16-position of progesterone (Fig. 7B) is exposed to the active iron center of CYP21A2. If k_{-c} and k_{2c} rates are slow (Fig. 7A) relative to k_c , then the ES' complex would build up over time, resulting in the burst phase kinetics. In the burst phase (initial rate of product formed vs. time in Fig. 6C, i.e. from time = 0 to time = t_f), there is more free enzyme available to form P, however, after a certain time t , the ES' complex builds up and occupies free enzyme, slowing down the rate (after time = t_f).

3.2.2. Rapid-Freeze Quench (Isolation of Compound I and Compound II)—

Although the rapid-freeze quench technique has not been employed with human steroidogenic cytochrome P450 enzymes, it is worthwhile to discuss the recent advances in this method to illustrate its potential application to study physiologically relevant enzymes.

The rapid-freeze quench variation of this technique has been used to measure the rate of formation of Compound I for CYP119 (Scheme 1, intermediate **G**) using spectroscopic methods (e.g. EPR, Mössbauer) [29]. In order to measure the iron active species by Mössbauer spectroscopy, the protein must be expressed using ^{57}Fe isotope [30]. In the quenching step after mixing the enzyme with *meta*-chloroperbenzoic acid to oxidize the heme iron, the complex was sprayed with liquid ethane (-183 C). In addition to Mössbauer spectroscopy measurements, EPR measurements may also be made to analyze the freeze-quenched samples to characterize the active iron species such as Compound I.

Additionally, Compound II (Scheme 1, intermediate **H**) has been spectroscopically measured using the CYP158 enzyme [31]. Compound II was formed as a result of hydrogen atom abstraction of nearby tyrosine groups by Compound I. When the tyrosine groups were mutated to phenylalanine, Compound II formation was decreased to 3% and Compound I was stable (90% yield). Furthermore, the $\text{p}K_{\text{a}}$ of Compound II ($\text{Fe}^{+4}\text{-OH}$) was measured to be ~ 12 , which is higher than the $\text{p}K_{\text{a}}$ values of axial histidine-ligated heme enzymes by 8.5 units. This difference in $\text{p}K_{\text{a}}$ suggests a possible explanation for different chemistry catalyzed by the two classes of enzymes (e.g. cysteine-ligated P450s catalyze $\text{RH} \rightarrow \text{ROH}$, and histidine-ligated peroxidases catalyze $\text{ROOR} \rightarrow 2\text{ROH}$). Additionally, the axial cysteine residue in P450 enzymes is the reason for the 450 nm Soret band upon CO binding [32]. If the axial cysteine residue is mutated to a histidine, then the Soret band appears at 420 nm [33].

3.2.3. Generation and Observation of Compound I from Hydroperoxide

Substrates—Compound I was trapped with EPR spectroscopy using bovine CYP11A1 purified from adrenal glands and a cholesterol hydroperoxide substrate (*20R*)-20-hydroperoxycholesterol in the absence of electron-transfer partners [34]. After addition of (*20R*)-20-hydroperoxycholesterol to the enzyme at room temperature, the mixture was frozen at 25 and 300 seconds with cold acetone at -60 C and characterized with EPR spectroscopy, which resulted in the observation of the $\text{Fe}^{+4}\pi$ -cation radical (Compound I). Over time, the hydroperoxide substrate had converted to (*20S*)-20,21-dihydroxycholesterol, which can be explained by 21-hydrogen atom abstraction by the Compound I generated *in situ*. The conversion of hydroperoxycholesterol derivatives to dihydroxycholesterol products was confirmed using the purified recombinant bovine CYP11A1 enzyme in a later report [35].

4. Kinetic Isotope Effects (Step 7, C-H Abstraction Step)

Physical organic chemists have employed kinetic isotope effects (KIEs) for decades to probe the mechanisms of reactions. Most commonly, deuterium is substituted for hydrogen at the site of interest, and the change in rate of the reaction is the KIE. The KIE derives from the difference in zero-point energy of a C-H bond versus a C- ^2H bond, due to the greater reduced mass and hence lower zero-point vibrational energy of the C- ^2H bond. Since the vibrational energy is essentially removed in a symmetrical transition state, the activation energy is greater for breaking the C- ^2H bond, and the rate of the reaction is reduced. The theoretical maximum of the deuterium KIE at 25 C is about 7, although much larger KIE

values can be observed if the mechanism involves proton-coupled electron transfer (hydrogen atom tunneling).

4.1. Intermolecular Kinetic Isotope Effect (DV , DV/K)

Consequently, deuterated P450 substrates allow for measurement of how rate-limiting the C-H abstraction step is in the overall catalytic cycle. Typically, a deuterated substrate is synthesized, and the rate of oxidation is tested over steady-state conditions. The $V_{\max}$ and $V_{\max}/K_m$ values are compared for the non-deuterated and deuterated substrates, and this measurement is termed intermolecular non-competitive KIE. For some steroid metabolizing enzymes with turnover numbers $\sim 10/\text{min}$, C-H bond breaking is significantly rate-limiting, leading to KIE values >2 . For human CYP21A2, the intermolecular KIE values for 21-hydroxylation of progesterone (Fig. 8, 1 or 1d) or 17-hydroxyprogesterone (Fig. 8, 4 or 4d) vary from 2–8 depending on substrate and enzyme system [28–36]. For human CYP17A1, the intermolecular KIEs are near 2 for the 17-hydroxylation of progesterone or pregnenolone [36].

4.2. Intramolecular Kinetic Isotope Effect (Metabolic Switching)

In some P450 enzymes, 2 or more different C-H site are hydroxylated, and deuterium incorporation at one of these sites often shifts reactivity to the non-deuterated positions, a process called metabolic switching. This phenomenon is quantitated as the intramolecular, non-competitive KIE and measured by comparing the product ratios derived from the deuterated and natural-abundance substrates.

For example, CYP21A2 has two different substrates: progesterone and 17-hydroxyprogesterone (Fig. 8, 1 and 4), and the 21-hydroxylated products are 11-deoxycorticosterone (**2**) and 11-deoxycortisol (**5**), respectively. Wild-type CYP21A2 also 16 α -hydroxylates $<1\%$ of progesterone, and CYP21A2 mutation V359A was engineered to 16 α -hydroxylate 40% of progesterone [37]. Thus, deuterium substitution at C21 or C16 can be used to probe for metabolic switching between C21 and C16 for CYP21A2 and its mutations. When [21,21,21- $^2\text{H}_3$]-progesterone (**1d**) was used as a substrate for CYP21A2, metabolic switching occurred where hydroxylation increased at the C16-position [36]. However, switching to C16 did not occur when 17-hydroxyprogesterone (Fig. 8, 4 or 4d) was used as the substrate. In contrast, for CYP21A2 mutation V359A, 16 α -hydroxyprogesterone was the major product for substrate **1d**, and the enzyme also 16 α -hydroxylated [21,21,21- $^2\text{H}_3$]-17-hydroxyprogesterone (**4d**) to [21,21,21- $^2\text{H}_3$]-16 α ,17-dihydroxyprogesterone (**6d**).

This difference in CYP21A2 hydroxylation at C21 and C16 for progesterone and 17-hydroxyprogesterone substrates can be quantitatively expressed as the intramolecular KIE (k_H/k_D). For wild-type CYP21A2, the intramolecular KIE at C21 with [21,21,21- $^2\text{H}_3$]-progesterone (**1d**) is 2.5, and for CYP21A2 mutation V359A, the intramolecular KIEs at C21 and C16 are 6.2 and 3.8, respectively—larger than the corresponding intermolecular KIEs [36]. Analogously, human CYP17A1 normally hydroxylates the 17- and 16 α -positions of progesterone in a 3:1 ratio, and CYP17A1 mutation A105L was engineered to reduce the progesterone 17:16 α -hydroxylation ratio to $\sim 9:1$ [38]. When the 17-position of progesterone

is regioselectively deuterated, the product ratios shift to 1:1 for wild-type CYP17A1 and to ~2:1 for mutation A105L, which yields intramolecular KIEs of 4.1 and 3.8, respectively [36, 39]. These experiments demonstrate how site-directed mutagenesis and isotopically labeled substrates are employed together in biophysical studies to understand the mechanisms of P450 catalysis.

5. Catalysis steps: Steady state rates ($E + S \rightarrow E + P$, $ES \rightarrow E + P$)

Conceptually, a pre-steady state experiment is designed similar to an initial burst study in order to measure the rate of the first turnover. This type of experiment can be expanded to study enzymes that perform sequential reactions, such as CYP17A1, where the product of the 17-hydroxylase reaction is the substrate for the 17,20-lyase reaction. The difference in this case is that an excess of enzyme is used to assure that only one turnover per enzyme molecule is measured [40]. Thus, pre-steady state, single-turnover experiments can be run with the enzyme and either the initial substrate, S^1 , or the intermediate, S^2 , where $[E] > [S^1]$ or $[E] > [S^2]$. Therefore, in the case where $[E] > K_d$, the $k_{\text{obs}} = k_2$ for S^1 and $k_{\text{obs}} = k_3$ for S^2 (Fig. 9). The experiment will be set up using a rapid chemical quench apparatus having preformed ES^1 or ES^2 complex in one syringe mixed with POR and NADPH in the second syringe. After some mixing time, the reaction is quenched to measure the intermediate or product formation [41].

From steady-state kinetic experiments (Michaelis-Menten), where the $d[ES]/dt$ is approximated to zero, we can calculate k_{cat} ($ES \rightarrow E + P$) and k_{cat}/K_m ($E + S \rightarrow E + P$). The enzymatic activities (k_{cat} values) of the six steroidogenic cytochromes P450 (CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP19A1, and CYP21A2) and other sterol metabolizing enzymes have been reported (Table 1). Because of the hydrophobic nature of steroids, the solubility of these substrates in experimental conditions is uncertain, and thus apparent K_m values must be interpreted with caution. For example, 2-hydroxypropyl- β -cyclodextrin has been used to solubilize lipophilic cholesterol in aqueous media in order to measure cholesterol-binding properties to P450 enzymes [42].

^d17,20-lyase activity only measured in the presence of cytochrome *b*₅

6. Multiple Step Reactions – Processivity

Several cytochrome P450 enzymes catalyze multiple step reactions. There are four steroidogenic P450 enzymes that catalyze reactions through more than one step:

- i. CYP11A1 (cholesterol to pregnenolone) – 22*R*-hydroxylation, then 20*R*-hydroxylation, followed by C20-C22 bond cleavage;
- ii. CYP11B2 (11-deoxycorticosterone to aldosterone) – 11 β -hydroxylation, followed by two successive hydroxylations at the 18-methyl position, yielding the 11 β ,18-hemiacetal;
- iii. CYP17A1 (pregnenolone to DHEA) – 17-hydroxylation followed by C17–C20 bond cleavage; and

- iv. CYP19A1 (androstenedione to estrone) – two hydroxylations on the 19-methyl position, followed by C10–C19 bond cleavage.

6.1. Processivity: Pulse-Chase Assays

Processivity refers to the sequential transformation of a substrate through 2 (or more) catalytic cycles without the release of the product from the first cycle (Fig. 10A). For a 2-step reaction, this terminology is equivalent to asking if the intermediate is released from the active site prior to the initiation of the second catalytic cycle. In addition to calculating the individual rate constants to measure the processivity, pulse-chase assays may be performed to directly test for processivity. The experiment is set up so that a labeled (i.e. radiolabeled or deuterated) substrate (S^1) is incubated with the enzyme, and after some time increment, a large amount of unlabeled intermediate (S^2) is added to the incubation (Fig. 10B). The amount of labeled product formed (P^*) is compared in the case where there was a chase added (addition of S^2 , Fig. 10B) to the case where there was no chase added (no S^2 , Fig. 10A). If the amount of labeled product (P^*) is substantially less when chase is added (Fig. 10D), then the reaction is said to be distributive (less processive), meaning that intermediate S^{2*} is primarily released before the second turnover and hence competes with unlabeled S^2 . If the amount of labeled product (P^*) is about the same with and without the chase (S^2) (Fig. 10C), then the reaction is processive, and the intermediate S^{2*} primarily remains bound in the active site and cannot be competed with unlabeled S^2 . Furthermore, the amount of unlabeled intermediate [S^2] can be varied to measure the concentration effects on the processivity of the enzyme. This experiment has been performed with CYP19A1 (aromatase) in the three step conversion of androstenedione to estrone [22]. CYP19A1 was found to be distributive with little or no processivity.

Interestingly, processivity has been measured in terms of observing intermediates by varying the interaction between the P450 and its redox partner. The cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone in three steps, via the sequential intermediates 22 R -hydroxycholesterol and 20 R ,22 R -dihydroxycholesterol. Contrary to expectations, the ratio of the intermediates to final product (pregnenolone) increases when the amount of the electron transfer partner FDX1 is reduced to subsaturating relative to P450 enzyme [52]. Similarly, in the case of CYP19A1, when the P450 enzyme was increased relative to the redox partner, POR, the production of 19-oxygenated androgen intermediates was increased [53]. These results suggest that processivity might be a function of redox partner abundance for some enzymes.

In addition, a combination of rapid kinetics and structural studies can provide models of substrate-specific processivity. Using pulse-chase experiments, zebrafish CYP17A1 has been shown to be more processive with the 5 -sequence pregnenolone to 17-hydroxypregnenolone to DHEA compared to the 4 -sequence progesterone to 17-hydroxyprogesterone to androstenedione [54]. A possible explanation for this increased processivity for pregnenolone versus progesterone is the presence of the hydrogen bond between serine-215 in the crystal structure of zebrafish CYP17A1 and the 3 β -hydroxy group of 5 -steroids but not the 3-ketone of 4 -steroids. As a result, the estimated ratio of turnover/dissociation rates for the 17-hydroxylated intermediates is ~0.1 for 17-

hydroxyprogesterone but ~2 for 17-hydroxypregnenolone [54]. In the crystal structure of human CYP17A1, asparagine-202 hydrogen bonds to both the 3 β -hydroxy group of 5-steroids and the 3-ketone of 4-steroids [55]; however, the dynamic properties of these interactions are not known.

6.2. Measuring Processivity from Single-Turnover Experiments

Processivity was also measured in terms of intermediate release rates using rapid chemical quench [22, 56]. Using single turnover conditions in which the amount of enzyme is about equal to the amount of substrate, the substrate, intermediate(s), and final product can be measured with a rapid chemical quench apparatus. The information gathered from this experiment can be useful in determining if there is a “lag” phase in the formation of the final product. If there is a lag phase in forming the final product, then the intermediates are freely dissociating from the enzyme active site, suggesting that the enzyme is distributive. In the aromatase study, the reaction monitored was the 3-step transformation: androstenedione $\rightarrow$ 19-hydroxyandrostenedione $\rightarrow$ 19-oxoandrostenedione $\rightarrow$ estrone. A lag phase was observed in the formation of the final product, estrone; this result from the single turnover assay was consistent with the results from the pulse-chase experiments (i.e. Fig. 10D).

7. Summary and limitations

Rapid kinetics techniques can dissect the rates of individual steps for P450-catalyzed reactions, including substrate binding, electron transfer, product release, and chemical steps. Combined with KIE experiments, these powerful methods can illuminate differences in enzymes and the reactions they catalyze, including the rate-limiting steps and processivity for sequential pathways. In some cases, observation of different “phases” in these rapid kinetics studies can reveal different conformations of the enzyme structure upon ligand [22] or redox partner [24] binding. Similarly, corresponding examples of structural changes in the P450 enzyme upon ligand binding have been shown through X-ray crystallography: substrate-free enzyme is in the “open” conformation whereas the substrate-bound enzyme is in the “closed” conformation [57].

The main limitation of studying individual steps of P450 enzymes is that a large amount of enzyme must be expressed and purified. One measurement on the stopped-flow apparatus requires up to 100 nmol of enzyme, so a good enzyme expression and purification system has to be established. This system may take at least a few months to establish the right strain of bacteria, plasmid, induction technique, and purification protocol. A second limitation is the availability of labeled substrates and intermediates, which might not be commercially available and require chemical synthesis and structural confirmation. A third limitation is access to a suitable rapid kinetics apparatus, which are expensive and difficult to maintain. Some institutions operate core facilities to increase access of investigators to the instrumentation. A fourth limitation is the capacity to perform the complex mathematical analysis of the data. Global fitting software such as DYNAFIT [58] are now available to determine rate constants from rapid kinetics data. The modeling program approximates the data to an equation to explain the behavior of the enzyme-catalyzed reaction and covers most enzyme systems commonly encountered. The final limitation is the creativity of the

investigator. We hope that this discussion will inspire enzymologists to consider innovative experiments to answer the vexing questions about how the P450 enzymes of steroid biosynthesis catalyze these essential and fascinating reactions.

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Abbreviations

CYP	Cytochrome P450
POR	P450 oxidoreductase (cytochrome P450 reductase)
cDNA	complementary DNA
FDX1	ferredoxin, ferredoxin reductase, FDXR
EPR	electron paramagnetic resonance
KIE	kinetic isotope effect
UV-Vis	ultraviolet-visible

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Highlights

- The cytochrome P450 reaction cycle has several microscopic steps
- Rapid-kinetics experiments permit the study of transient intermediates
- Single-turnover experiments determine if product release is rate-limiting
- A kinetic isotope effect is observed if C-H bond breaking is rate-limiting

Figure 1.

(A) A cartoon showing the cytochrome P450 heme active site (reduced or ferrous, Fe⁺²) bound to CO ligand. (B), (C), (D): Frontier molecular orbital interaction between the d orbitals of Fe and the p* antibonding molecular orbitals of CO (B and C), or sp-molecular orbital of CO and the d_{z2} orbital of Fe (D). gr1

Figure 2.

(A) Cartoon of spectral changes (UV-Vis spectrum) of the heme center with different conditions. Absorption maxima of the Soret band are indicated in parentheses. (B) “Type I” binding difference spectrum. (C) “Type II” binding difference spectrum. For experimental data see reference [18]. The reduction from Fe³⁺ (ferric) to Fe²⁺ (ferrous) forms as shown in spectra [iv] and [iii], respectively, is achieved via addition of solid sodium dithionite.gr2

Figure 3.

Energy diagrams of the low-spin Fe d orbitals of (A) an octahedral ferric (Fe^{+3}) complex (five d -orbital electrons), (B) after axial distortion, and (C) after rhombic distortion. P450 enzymes have been shown to be in the rhombic state based on EPR spectroscopy [20·21].gr3

Figure 4.

Setup for stopped-flow studies to determine binding rates of substrate with enzyme to form the [ES] complex. Enzyme structure is of the human placental aromatase (CYP19A1, PDB: 3EQM), and the experimental design was used to measure the rates of step 1 (Scheme 1).gr4

Figure 5.
Setup for the rapid chemical quench apparatus. Structure of human placental aromatase (PDB: 3EQM). Structure of CPR: Rat NADPH-Cytochrome P450 Reductase (PDB: 3WKT).gr5

Figure 6.

(A) An enzyme-catalyzed single-step reaction including substrate binding (k_1), product formation (k_2), and product release (k_3). (B) The plot of product formed with respect to time in the absence (B), and presence (C) of a burst. The point t_1 is the time required to form one molecule of product per molecule of enzyme.gr6

Figure 7.

(A) Illustration of a conformational change in the ES complex of CYP21A2. (B) The ES' complex is proposed to be the complex giving rise to the 16 α -hydroxyprogesterone product. P1: 11-deoxycorticosterone (21-hydroxyprogesterone), P2: 16 α -hydroxyprogesterone.gr7

Figure 8.

The reactions catalyzed by wild-type CYP21A2 with (A) progesterone substrate (**1**or**1d**) and (B) 17-hydroxyprogesterone substrate (**4**or**4d**).gr8

Figure 9.

Individual rate constants that can be measured of a two-step reaction through experiments discussed in the text. pre-SS: pre-steady state.gr9

Figure 10.

An illustration of a pulse-chase experiment of an enzyme that catalyzes a successive 2-step reaction. (A) The reaction starting with labeled substrate (S^1) that occurs without adding an unlabeled intermediate. (B) An experiment with labeled substrate (S^1) where a chase of unlabeled intermediate (S^2) is added at time t. Theoretical results obtained from a (C) processive enzyme and a (D) distributive enzyme. $[P^*]_{(A)}$ is the amount of product detected without a chase (S^2) added as shown in (A) and $[P^*]_{(B)}$ is the amount of labeled product detected in (B).gr10

Scheme 1.

Catalytic cycle of P450 catalyzed C-H hydroxylation reaction.sc1

Table 1Reported k_{cat} and apparent K_{m} values for human cytochrome P450 reactions in steroid and sterol metabolism.

Enzyme	Substrate → Product	k_{cat}	K_{m}	ref
CYP3A4	testosterone → 6 β -testosterone	1.3 s ⁻¹	23 μ M	[43]
CYP7A1	cholesterol → 7 α -hydroxycholesterol	3.1 s ⁻¹	1.3 μ M	[44]
CYP11A1 ^a	cholesterol → pregnenolone	0.021 s ⁻¹	14 μ M	[45]
CYP11A1	20R,22R-dihydroxycholesterol → pregnenolone	4.1 s ⁻¹	10 μ M	[46]
CYP11B1	11-deoxycorticosterone → corticosterone	1.35 s ⁻¹	--	[47]
CYP11B1	11-deoxycortisol → cortisol	1.61 s ⁻¹	--	[47]
CYP11B1	corticosterone → 18-hydroxycorticosterone	0.008 s ⁻¹	--	[47]
CYP11B2	11-deoxycorticosterone → corticosterone	0.63 s ⁻¹	--	[47]
CYP11B2	corticosterone → aldosterone	0.011 s ⁻¹	--	[47]
CYP17A1 ^b	pregnenolone → 17-hydroxypregnenolone	0.037 s ⁻¹	0.65 μ M	[48]
CYP17A1 ^{c (+b₅)}	pregnenolone → 17-hydroxypregnenolone	0.065 s ⁻¹	0.65 μ M	[48]
CYP17A1 ^b	progesterone → 17-hydroxyprogesterone	0.056 s ⁻¹	1.0 μ M	[48]
CYP17A1 ^{c (+b₅)}	progesterone → 17-hydroxyprogesterone	0.078 s ⁻¹	1.0 μ M	[48]
CYP17A1 ^{c,d (+b₅)}	17-hydroxypregnenolone → DHEA	0.041 s ⁻¹	1.2 μ M	[48]
CYP17A1 ^{c,d (+b₅)}	17-hydroxyprogesterone → androstenedione	0.030 s ⁻¹	5.0 μ M	[48]
CYP19A1	androstenedione → estrone	0.060 s ⁻¹	44 nM	[22]
CYP19A1	19-oxoandrostenedione → estrone	0.42 s ⁻¹	18 μ M	[22]
CYP21A2	progesterone → 11-deoxycorticosterone	2.8 s ⁻¹	0.21 μ M	[28]
CYP21A2	17-hydroxyprogesterone → 11-deoxycortisol	4.0 s ⁻¹	1.5 μ M	[28]
CYP24A1	1,25-dihydroxyvitamin D3 → 1,24,25-trihydroxyvitamin D3	0.44 s ⁻¹	--	[49]

Only the separate k_{cat} and K_{m} values are considered and not the ratio $k_{\text{cat}}/K_{\text{m}}$ [50–51]; DHEA, dehydroepiandrosterone^a rate of a 3-step reaction^b without cytochrome *b*₅^c with cytochrome *b*₅