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Review

# 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 discreet 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 1000 $s^{-1}$, 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.



**Contents**



*Abbreviations:* CYP, cytochrome P450; POR, P450 oxidoreductase (cytochrome P450 reductase); cDNA, complementary DNA; FDX1, ferredoxin; FDXR, ferredoxin reductase; EPR, electron paramagnetic resonance; KIE, kinetic isotope effect; UV–vis, ultraviolet–visible.

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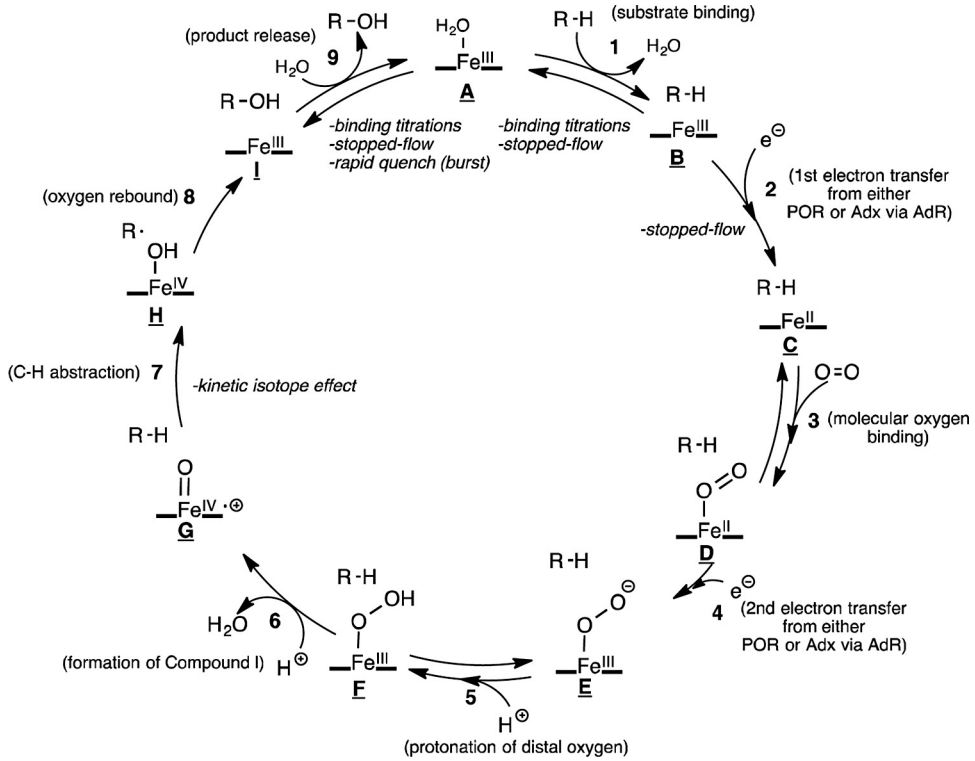
## 1. Cytochrome P450 enzymes

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, P450scc, cholesterol side chain cleavage enzyme), CYP11B1 (P450 11B1, P450c11β, steroid 11β-hydroxylase), and CYP11B2 (P450 11B2, P450c11AS, aldosterone synthase), as well as the 3 microsomal enzymes CYP17A1 (P450 17A1, P450c17, steroid 17α-hydroxylase/17,20-lyase), CYP21A2 (P450 21A2, P450c21, steroid 21-hydroxylase), and CYP19A1 (P450 19A1, P450aro, 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: $1/k_{ss} = \Sigma_{(n=i)} 1/k_i$, 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].




**Scheme 1.** Catalytic cycle of P450 catalyzed C—H hydroxylation reaction.