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Review

# Single-molecule enzymology of steroid transforming enzymes: Transient kinetic studies and what they tell us

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ABSTRACT

Structure-function studies on steroid transforming enzymes often use site-directed mutagenesis to inform mechanisms of catalysis and effects on steroid binding, and data are reported in terms of changes in steady state kinetic parameters $k_{cat}$,$K_m$ and $k_{cat}/K_m$. However, this dissection of function is limited since $k_{cat}$ is governed by the rate-determining step and $K_m$ is a complex macroscopic kinetic constant. Often site-directed mutagenesis can lead to a change in the rate-determining step which cannot be revealed by just reporting a decrease in $k_{cat}$ alone. These issues are made more complex when it is considered that many steroid transforming enzymes have more than one substrate and product. We present the case for using transient-kinetics performed with stopped-flow spectrometry to assign rate constants to discrete steps in these multi-substrate reactions and their use to interpret enzyme mechanism and the effects of disease and engineered mutations. We demonstrate that fluorescence kinetic transients can be used to measure ligand binding that may be accompanied by isomerization steps, revealing the existence of new enzyme intermediates. We also demonstrate that single-turnover reactions can provide a $k_{lim}$ for the chemical step and $K_s$ for steroid-substrate binding and that when coupled with kinetic isotope effect measurements can provide information on transition state intermediates. We also demonstrate how multiple turnover experiments can provide evidence for either "burst-phase" kinetics, which can reveal a slow product release step, or linear-phase kinetics, in which the chemical step can be rate-determining. With these assignments it becomes more straightforward to analyze the effects of mutations. We use examples from the hydroxysteroid dehydrogenases (AKR1Cs) and human steroid 5β-reductase (AKR1D1) to illustrate the utility of the approach, which are members of the aldo-keto reductase (AKR) superfamily.


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## 1. Introduction

To the novice, enzymes are described in terms of their steady state kinetic parameters: $k_{cat}$ ($V_{max}/[E_t]$) or turnover number in units of $min^{-1}$; $K_m$ (substrate concentration at which half-maximal velocity is observed) in units of molarity; and $k_{cat}/K_m$ or catalytic

*Abbreviations:* AKRs, aldo-keto reductases; DHT, 5α-dihydrotestosterone; 3α-diol, 5α-androstane-3α,17β-diol; KIE's, kinetic isotope effects; HSDs, hydroxysteroid dehydrogenases.

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efficiency in units of $min^{-1} M^{-1}$ making it a formal bimolecular rate constant. Both $k_{cat}$ and $K_m$ are macroscopic rate constants and can comprise multiple steps governed by several microscopic rate constants that are not revealed in these terms. Moreover, $K_m$ is often misinterpreted as being a measure of substrate affinity when even the simple Briggs–Haldane [1] treatment of the Michaelis–Menten equation [2] teaches us otherwise. Nevertheless the literature is abound with articles that interpret the effects of disease-based mutations or the effects of site-directed mutants on steroidogenic enzymes using these steady-state kinetic parameters when the true effect of these mutations is buried within the microscopic rate constants that make up these terms. The cursory investigation of mutations using steady-state kinetic parameters can occur when investigators interested in the physiology of a problem do not appreciate the shortcomings of the approach. Clearly, there is a need for approaches that permit individual steps governed by microscopic rate constants in the reaction scheme to be dissected so that the true effect of a mutation can be revealed. Transient kinetic approaches in which discrete steps of an enzyme reaction are captured provide the necessary window to dissect enzyme reactions and the effects of mutations more precisely. The use of transient kinetics is clearly beneficial to the study of steroid transforming enzymes, where natural occurring mutations are often causal in disease [3–6] and site-directed mutagenesis can inform structure-function relationships. As will be seen later one criteria for this approach is that it requires milligram amounts of homogeneous enzyme and therefore precludes the studies of some hydroxysteroid dehydrogenases. However, with improvement in expression systems and enzyme purification techniques this hurdle will likely be surmounted in the future.

## 2. Stopped-flow instrument/quench flow

Stopped-flow spectrometry provides a method to dissect individual components of an enzyme dependent reaction. Stopped-flow techniques were developed by Britton–Chance in the Johnson Foundation at the University of Pennsylvania [7,8]. The essentials of the apparatus consist of (a) two pneumatically driven syringes which contain different components of the reaction under study (enzyme and substrate); (b) a mixing device; (c) an observation cell with detection system; and (d) a stopping syringe. The reactant syringes deliver set amounts of reactant into the mixing chamber (20–50 μL) and the mixture is forced into the observation chamber. A small movement of the plunger of the stopped syringe brings it to a mechanical stop which prevents further mixing and activates the detection system. The time that elapses between mixing the reactants and data collection is known as the dead-time of the instrument which is typically 1–3 ms. The resultant absorbance or fluorescence change that occurs as a result of the enzymatic reaction is measured on a millisecond time scale. The experimental set up requires that a change occurs that can be measured spectrally, Fig. 1. When a spectral change cannot be monitored the alternative would be to conduct quench-flow experiments. Data from quench-flow experiments can be analyzed in the same manner as data from stopped-flow experiments since the transient kinetics being monitored are the same. In the stopped-flow, different types of experiments can be conducted. These include ligand binding studies, ligand chase experiments, single-turnover experiments and multiple turnover experiments. Single-turnover experiments allow only a single turnover event to occur and is representative of a single molecule of substrate being turned over by a single molecule of enzyme and can be referred to as “single-molecule enzymology.” Examples of these experimental approaches will be given in subsequent sections.

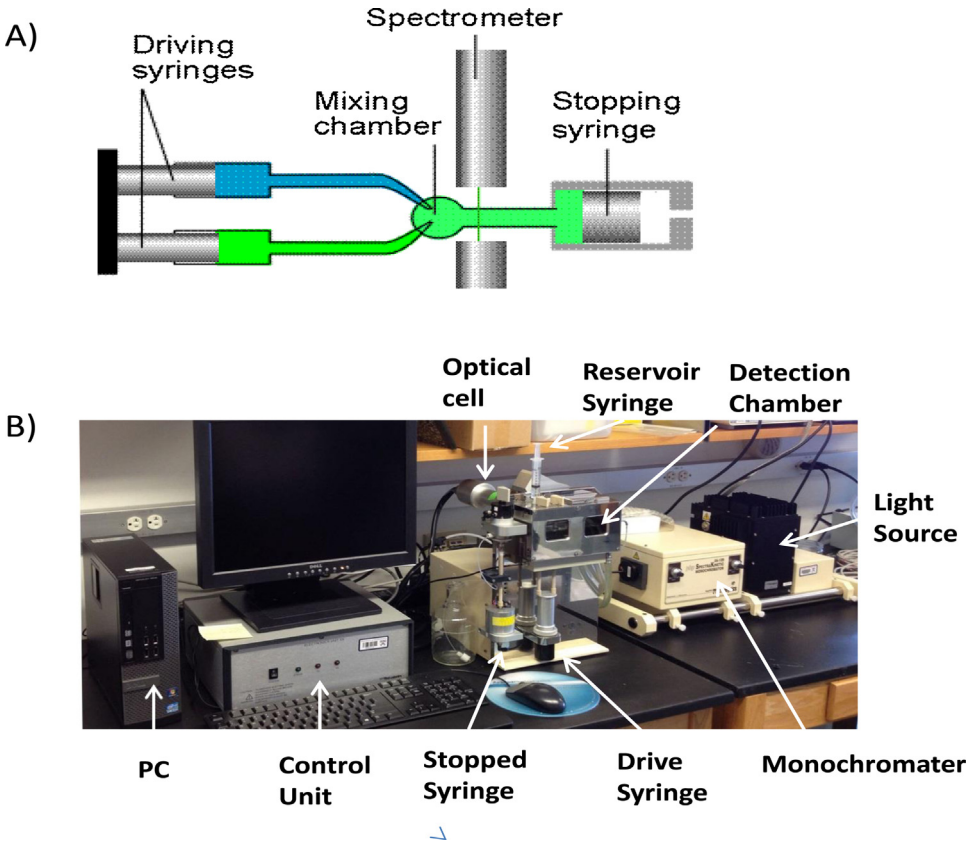


**Fig. 1.** Stopped-flow instrument. (A) Schematic of stopped-flow principle; (B) Components of Applied Photophysics stopped-flow instrument.