

Enzyme Assays and Kinetics

Arthur L. Haas, Ph.D.

Department of Biochemistry and Molecular Biology

Review: Garrett and Grisham, "Enzyme Kinetics" (Chapt. 14), in *Biochemistry*, Second Edition, Saunders, Fort Worth, 1999.

Suggested additional reading: Scopes, R.K. *Protein Purification: Principles and Practice*, Springer-Verlag, New York, Chapt.8, 1982.

Contemporary bioscience is ultimately interested in understanding molecular interactions between the various components that comprise the cell. These binding events invariably result in some *response* whose quantitation can be exploited as a **reporter function** to characterize the magnitude of the interaction and changes resulting from genetic manipulation and/or post-translational modification. Therefore, the central principles of enzyme assays and kinetic analysis become equally applicable for the quantitative evaluation of systems not usually considered in classic enzymology.

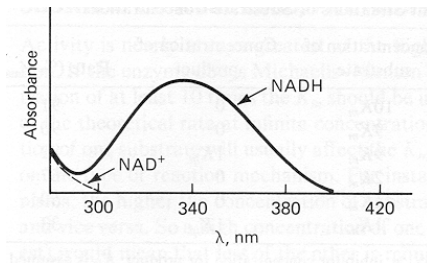
I. Properties of enzyme assays

A. Enzyme assays require measurements of product formation or substrate disappearance over time in order to determine the **initial rate (v_0)** for the process under consideration. Because of the problem of **signal to noise ratio** during the measurement of an initial rate, it is generally preferable to measure product appearance rather than substrate disappearance.

B. Two basic types of assays can be used in measuring rates.

1. In **continuous assays** the reaction is monitored in real time and v_0 is determined directly as a slope of $\Delta P/\Delta t$ (or $-\Delta S/\Delta t$). Such assays require some physical technique to continuously monitor product formation or substrate loss that exploits a difference in signal between the two species. Spectroscopic methods are frequently used in continuous assays since these operate in real time and are non-destructive to the sample.

Example: Enzymes utilizing the cofactor **Nicotinamide Adenine Dinucleotide (NAD⁺)** can be readily quantitated in continuous assays by exploiting the difference in absorbance between NAD⁺ and its product **NADH**, as illustrated by the reaction of lactate dehydrogenase.



Spectrophotometric assays rely on the direct proportionality between absorbance and concentration of the **chromophore**, which is formally expressed by **Beer's Law**.

2. More frequently, quantitation of the product or substrate can only be made by destructive methods that preclude the continuous monitoring of the reaction's time course. In these cases v_o can be estimated by the use of a **stopped assay** in which the reaction is allowed to proceed for a specific amount of time then **quenched** to prevent further reaction so that product formed or substrate remaining can be analyzed. Because we wish to approximate v_o , stopped assays rely on the assumption that *the rate is linear over the time of the measurement*. This assumption should always be verified by conducting at least one experiment at different times to confirm linearity of the reaction. Nonlinearity of the time course frequently results in *underestimation of v_o* .

Practical considerations:

Timing-

Effective mixing and quench-

Time points-

Lag effects-

Thermal equilibration-

3. Both kinetic assays methods lend themselves to a third type of assay in which the reaction is allowed to proceed to completion. Such **end point assays** cannot be used to study the kinetics of the process nor assay enzyme activity but, rather, are used to quantitate total substrate under the assumption that $S_0 = P_4$.

4. Physical measurements yield values for **relative quantitation** (e.g., absorbance with spectrophotometry, counts per minute (cpm) with radiolabels, arbitrary units with densitometry) that require a value for **specific yield** to allow **absolute quantitation**. For spectrophotometry the **molar extinction coefficient** (ϵ) for the chromophore is a form of specific yield.

With radiolabels the specific yield is termed the **specific radioactivity**, expressed as Curies/quantity (e.g., mCi/mol, Cu/mmol, etc.).

Densitometry of gels or autoradiograms requires comparison to a series of standards for exact quantitation since the specific yield may vary considerably with the conditions of the experiment. X-ray film used in autoradiography is even more problematic since the **dynamic range** of film is narrow and non-linear.

5. Frequently the product of the reaction under consideration cannot be readily detected, requiring a **coupled assay**. Coupled assays can also be used when the actual substrate is unstable and therefore must be generated during the assay. The validity of coupled reactions depend on the fact that rate assays *can only detect the slowest or rate limiting step*. Therefore, if the coupling steps are faster than the reaction under consideration, they are undetected.

II. Single substrate kinetics

Enzyme kinetics can be a powerful tool in providing quantitative evidence for an hypothesis. However, it is essential to always keep in mind that ***kinetics can never unequivocally prove a model*** (since it is always possible to conceive of an alternative model that also satisfies the experimental data) but ***kinetics does allow one to rule out models whose predictions do not fit the experimental data.***

- A. Enzymes that bind only one substrate conform to **single substrate kinetics**. The kinetic scheme for these enzymes involves a formation of a single **ES** or **Michaelis complex** that proceeds to product.

For most enzymes, substrate binding is near the **diffusion limited rate**; therefore, the slowest step must be that involving catalysis, defined as the **catalytic rate constant (k_{cat})** or **turnover number**. [Note though that some enzymes have their rate limiting step as product release, usually due to a significant conformational change]. The first order rate constant for dissociation of the substrate from the active site varies considerably since this is the term most critical in determining the overall K_d for substrate. The relative magnitudes of the opposing rate constants for dissociation *versus* catalysis result in two extreme cases:

1. **Pre-equilibrium mechanisms-** For those enzymes in which dissociation of ES is faster than the catalytic step, binding of substrate is in pre-equilibrium and the resulting K_m is equal to K_d (the original model of Henri, Michaelis, and Menten). Most enzymes can be described by this **pre-equilibrium assumption**. It is critical to keep in mind that substrate is rapidly binding to and diffusing off the enzyme in these systems with only a fraction of the binding events being trapped as product. Therefore, K_m in this case is an equilibrium constant identical to the $K_{dissociation}$ (K_d) for the binding step and can be used to calculate ΔG° for binding using the **Gibbs free energy equation**.

2. **Steady state mechanisms-** For those enzymes having either very slow off rates (and correspondingly high affinities) or unusually fast catalytic steps, the substrate within the ES complex proceeds to product faster than it can escape from the catalytic site by dissociation (the later model of Briggs and Haldane). For these enzymes K_m is not equivalent to the equilibrium constant K_d and cannot be used to calculate the ΔG° , but rather is a ratio of rate constants.

In general we cannot know *a priori* whether a given enzyme follows Henri-Michaelis-Menton or Briggs-Haldane kinetics. Both models produce the same mathematical model and differ only in their interpretations of the exact meaning of K_m . In general, one assumes the pre-equilibrium case unless inconsistencies in the experimental data argues otherwise.

- B. Since the full kinetic scheme contains five variables ($[E]$, $[S]$, $[ES]$, $[EP]$, and $[P]$) a unique solution cannot be derived without reducing the scheme to a single variable. This is accomplished by making certain **simplifying assumptions** for which *the resulting mathematical expression is only valid under conditions for which the assumptions are valid:*

1. Immediately after mixing the enzyme establishes an equilibrium for substrate binding (pre-equilibrium assumption) or achieves steady state (steady state assumption).

2. The initial substrate concentration is very much greater than initial enzyme concentration ($[S_0] \gg [E_0]$).

3. Only the initial velocity (v_0) is measured.

C. The resulting equation is that of a rectangular hyperbola defining a mathematical function that asymptotically achieves V_{\max} at infinite substrate concentration.

- D. The **double reciprocal** or **Lineweaver-Burke plot** transforms the hyperbolic Henri-Michaelis-Menten equation into a linear form from which K_m and V_{max} can be graphically determined. Several other linearized forms of the hyperbolic equation (such as the Dixon plot) are in use, each of which attempts graphically to address the inherent data weighting problems at low substrate concentration.

Exact values of k_{cat} cannot be calculated from V_{max} without knowing the concentration of active enzyme ($[E]_o$); however, lower limit approximations for k_{cat} can be made by making the additional assumption that all enzyme protein is active enzyme protein (almost never the case).

In *lieu* of an exact value for $[E]_o$ we can express enzyme activity in **International Units (I.U.)**, defined as *the quantity of enzyme producing 1 micromole of product per minute under optimal conditions* (saturating substrate and other parameters such as pH, temperature, buffer/salt conditions that must be defined). Other units can also be used when appropriate but must be thoroughly defined.

Enzyme purity is expressed as **specific enzyme activity**, defined as I.U./mg protein.

III. Simple inhibition in single substrate systems

- A. **Competitive inhibition** results when an enzyme can bind substrate or inhibitor but not both simultaneously (or when binding of one blocks the binding of the other either by steric hindrance or conformational change).

B. **Noncompetitive inhibition** results when the enzyme can bind both the substrate and inhibitor at the same time.

IV. Two substrate kinetics

Most enzymes require the binding of two substrates during their catalytic cycles. Three general cases arise based on the relative affinities for binding of each ligand.

A. **Random substrate addition** results when the enzyme shows no preference for the order of substrate binding. The relationship among the different K_d values is constrained by thermodynamics.

B. Ordered substrate addition results when the enzyme exhibits an absolute requirement for binding of one substrate (the **leading substrate**) prior to binding of the second substrate (the **trailing substrate**). Ordered substrate addition is an extreme case of random addition and usually (but not always) implies the requirement for a conformational change following binding of the leading substrate to create the binding site for the trailing substrate.

C. **Random binding with preferred order** results in random addition systems for which there is a considerable difference in affinity for a substrate when it is the leading versus trailing substrate.

- D. **Ping Pong kinetics** results when binding of the leading substrate results in a catalytic step that produces a covalent enzyme intermediate that is subsequently transferred to the trailing substrate. Ping pong kinetics are the defining feature of enzymes that function through **covalent catalysis**.