Methods in Enzymology

Volume 63

Enzyme Kinetics and Mechanism

Part A
Initial Rate and Inhibitor Methods

EDITED BY

Daniel L. Purich

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Preface

In the early years of chemistry the identification of new reactions preceded serious consideration of reaction kinetics, and it was not until Berthelot derived the bimolecular rate equation in 1861 that chemical kinetics offered any real value to the practicing chemist. Fortunately, biochemistry, which had its roots in the late nineteenth century, experienced the benefit of developments in kinetic theory. In fact, kinetic arguments have played a major role in defining the metabolic pathways, the mechanistic action of enzymes, and even the processing of genetic material. Nevertheless, it is amusing to witness the disdain of many investigators toward mechanistic conclusions drawn from kinetic data. After all, kinetic arguments are frequently tediously detailed with algebra and calculus, and so many refuse to believe that such abstract constructs truly apply to real systems. For those of us who derive much fascination, excitement, and satisfaction from the combination of chemical and kinetic probes of enzyme mechanism and regulation, the statement that "kinetics never proves anything" is especially amusing. When one views the definition of the word "proof" as an operation designed to test the validity of a fact or truth, the preceding statement serves only to demonstrate that we have failed to communicate the power and scope of kinetic arguments. The purpose of this volume is to initiate those who are interested in an advanced treatment of enzyme kinetic theory and practice. Indeed, this area of biochemistry is rich in information and experimental diversity, and it is the only means to examine the most fundamental characteristic of enzymes—catalytic rate enhancement.

Parts A (Volume 63) and B (Volume 64) are the first of a series of volumes to treat enzyme kinetics and mechanism, and the chapters presented have been written to provide practical as well as theoretical considerations. However, there has been no attempt on my part to impose a uniform format of symbols, rate constants, and notation. Certainly, uniformity may aid the novice, but I believe that it would also present a burden to those wishing to examine the literature. There, the diversity of notation is enormous, and with good reason, because the textural meaning of particular terms must be considered. In this respect, the practice of utilizing a variety of notations should encourage the student to develop some flexibility and thereby ease the entry into the chemical literature of enzyme dynamics and mechanism. Each of the contributors is an expert in the literature, and I have been especially pleased by the constant reference to key sources of experimental detail.

I wish to acknowledge with pleasure and gratitude the cooperation

and ideas of these contributors, and I am indebted in particular to Professors Fromm and Cleland for many suggestions during the initial stages of developing the scope of this presentation. My students, certainly R. Donald Allison, also deserve much praise for surveying the literature and convincing me that a balanced view of the field may be presented in the confines of this series. The staff of Academic Press has also provided great encouragement and guidance, and to them I am deeply indebted. Finally, I wish to acknowledge the wisdom and friendship offered to me by Sidney Colowick and Nathan Kaplan.

DANIEL L. PURICH

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Section I

Initial Rate Methods

[1] Practical Considerations in the Design of Initial Velocity Enzyme Rate Assays

By R. DONALD ALLISON and DANIEL L. PURICH

Developing a reliable initial velocity enzyme assay procedure is of prime importance for achieving a detailed and faithful analysis of any enzyme. This objective is quite different from the use of enzyme assays in enzyme purification or clinical chemistry, where the focus is on estimates of the enzyme content of various samples. In that case, one is particularly concerned with optimizing assay conditions by including substrates, cofactors, and activators at optimal (often saturating) levels and with minimizing interfering agents. Thus, the emphasis is on determining enzyme concentration in a routine, easy, and reproducible fashion. On the other hand, the enzyme kineticist must often work at subsaturating substrate and effector levels to evaluate the rate-saturation behavior. When two or more substrates are involved, the problem of obtaining initial velocity data becomes more considerable. This chapter treats of the practical aspects of initial rate enzyme assay.

General Experimental Design

The initial rate phase of an enzymic reaction typically persists for 10 sec to several hundred seconds. Thus, various methods including spectrophotometry, radioactive assay, and pH-stat procedures may be used along with manual mixing and manipulation of samples. Prior to addition of the enzyme (or one of the substrates) to initiate the reaction, the assay sample (usually in 0.05–3.0 ml volumes) is preincubated at the reaction temperature for several minutes to achieve thermal equilibration, and a small aliquot of enzyme is added to initiate the reaction. The increase in the product concentration or the drop in substrate concentration may then be measured. The basic goals are to initiate the reaction in a manner that leads to immediate attainment of the initial velocity phase and to obtain an accurate record of the reaction progress.

For most enzyme rate equations to apply to real systems, one must be certain that the conditions placed upon the mathematical derivation are satisfied in the experiment. Since rate equations become quite complex as product accrual becomes significant, the initial rate assumption is frequently taken to linearize the equations. Experimentally, one draws the tangent to the reaction progress curve as shown in Fig. 1. The best estimates of the slope of this line will be obtained from the most complete

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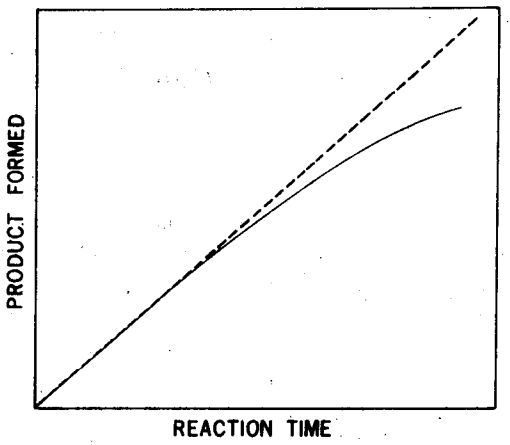

FIG. 1. Plot of product formation versus reaction time for an enzyme-catalyzed reaction. The solid line represents the reaction progress, and the dashed line is the tangent to the curve at low product formation. This tangent is the initial velocity, and it is expressed in units of molarity per minute.

record of the initial rate phase, and continuous assays are thus preferable to single-point assays (see below). The duration of the linear initial rate phase depends upon many factors, including the equilibrium constant, the fractional saturation of the enzyme with substrate(s) and product(s), the buffering capacity of the medium, and the concentration ratio of the least abundant substrate relative to the enzyme. Below a $[S]_{total}/[E]_{total}$ value of 100, the steady state may not persist for long, and nonlinear initial rates will frequently be observed. In some cases, the rate may appear linear, but virtual linearity should not be the only criterion used in establishing reaction conditions. With conditions such as a very favorable equilibrium constant, no product inhibition, and a high $[S]_{total}/[E]_{total}$ value, the initial reaction velocities can be maintained for a considerable period of time.

The Initial Rate Condition

As a general guideline, one assumes that the initial rate persists for a period of time during which the substrate(s) concentration is within 10% of the initial value. This is probably true only for reactions that are thermodynamically quite favorable, and even so it is best to choose an assay method that is safely within this range. Nonetheless, there is no *a priori* guarantee that product inhibition will not account for a significant error in the estimation of initial rates, and tests should be made for even the most favorable reactions (see below). Since the equilibrium constant for the

particular reaction will presumably be known, one may estimate the extent of the reaction by the following simple expression:

$$K_t' = \frac{(P_0 + x)(Q_0 + x)(R_0 + x) \ldots}{(A_0 - x)(B_0 - x)(C_0 - x) \ldots} \quad (1)$$

where K_t' is the apparent product: substrate ratio (or "mass action ratio") at time t, x is the concentration change measured at the midpoint of the experimental assay (i.e., time t), and A_0, B_0, C_0, P_0, Q_0, and R_0 are the initial substrate and product concentrations. If it is found that K_t' is not much different from the apparent equilibrium constant (K') for the reaction, then one must reduce x by use of a more sensitive assay. Let us consider the case of yeast hexokinase, where the apparent equilibrium constant ($K' = 4.9 \times 10^3$ at pH 7.5) is quite favorable. Assuming we have both the glucose and ATP concentrations at 0.1 mM (i.e., near their Michaelis constants), then a 5% conversion would yield a K_t' of 2.8×10^{-3}, suggesting that the system is quite far from equilibrium. On the other hand, we may consider the acetate kinase reaction (written in the direction of acetyl phosphate formation where $K' = 3.3 \times 10^{-4}$ at pH 7.4). At an acetate concentration of 10 mM and an ATP level of 1 mM, a 5% conversion of substrate to products would yield a K_t' of 2.7×10^{-4}, not far away from the equilibrium value. In this case, it would be advisable to reduce the percentage of substrate conversion in the rate assay. An obvious extension of these comments is that the deviation from initial rates may be a greater problem in some product inhibition studies.

It is not certain that product accumulation during the "initial rate" will lead to insignificant error even when substrate conversion is quite low. Indeed, for some systems the inhibition constants for a particular product may be quite low. Consider the brain hexokinase reaction where $K_{\text{glucose-6-P}}$ is approximately 10^{-5} M[1] but the K_{glucose} is about 5-fold higher. Another case in point is the PRPP:ATP phosphoribosyltransferase from *Salmonella typhimurium;* here N^1-phosphoribosyl-ATP has a dissociation constant of 3.8×10^{-6} M, but the affinity for either substrate is considerably lower.[2] The reduced coenzyme in NAD^+-dependent dehydrogenases is frequently a potent inhibitor as well. One strategy around the problem of product accumulation is to remove the product by use of an auxiliary enzyme system (see this volume [2]). This can be especially useful when the auxiliary system also serves to regenerate one of the substrates. For example, the pyruvate kinase/lactate dehydrogenase coupled assay for kinases maintains the initial ATP concentration, and it also provides for a

[1] J. Ning, D. L. Purich, and H. J. Fromm, *J. Biol. Chem.* **244**, 3840 (1969).
[2] J. E. Kleeman and S. M. Parsons, *Arch. Biochem. Biophys.* **175**, 687 (1976).