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Rapid Report

Determination of the maximum velocity and Michaelis constant of enzymes by a fixed-point method which avoids the necessity to measure initial rates

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Measuring the initial velocity is difficult in some enzyme assays where a significant fraction of the substrate is consumed. Here a solution to this problem is proposed; the time to produce a fixed amount of reaction product is measured. This time is inversely proportional to the initial velocity, and is related to the maximum velocity and Michaelis constant by a simple equation and linear plot. The method is illustrated using the reaction catalysed by pyruvate kinase.

The usual method for measuring enzymic activity is based on the determination of the initial rate (v_0); that is, the rate measured after the initial pre-steady-state period is over, but before there has been substantial substrate utilisation and product accumulation. The ease with which these rates can be determined depends on the sensitivity of the assay method and sometimes a significant fraction of the substrate may be used by the time that measurements are made. Under these conditions it would be very easy to underestimate the v_0.

These rate measurements usually have one of three specific purposes: (1) to measure the quantity of the enzyme which is present; (2) to determine the effect of alterations in the assay environment on the enzyme; or (3) to study the kinetic properties of the enzyme. This latter type of study would usually involve determining the maximum velocity (V_m) and Michaelis constant (K_m) only, although more extensive kinetic studies are frequently performed. In the first two cases A_0 may often be chosen so as to simplify measurement of v_0. However, in the third case, the experiments necessarily involve variations of A_0 over a range may not be freely chosen by the investigator; rather it is dictated by the properties of the enzyme. Unless a sensitive assay is available, there is sometimes no choice but to try to estimate the v_0 over a period where a significant fraction of the substrate has been used up. Moreover, this fraction may vary depending on A_0 so that some rate measurements are more likely to be biased than others. In this report I will be concentrating on the particular problems associated with experiments to determine V_m and K_m.

Various alternatives to simplify or avoid the problem of measuring v_0 have been suggested. These include measuring chords [1–4], estimating v_0 from the shape of the reaction progress curve [5,6] or determining the half-time [7] (i.e. the time for the reaction to reach half completion). None of these methods seems to be used widely. In this report I will propose a variation on the half-time method [7] in which the time to reach a predetermined product concentration is measured. This method will be referred to as the 'fixed-point' method.

The basic idea behind the present method is very simple and is based on Eqn. 1, the integrated Michelis-Menten equation which describes the amount of product (z) formed in an irreversible, single-substrate enzyme-catalysed reaction exhibiting no inhibition by accumulating products.

$$V_m \cdot t = z - K_m \cdot \ln(1 - z/A_0) \quad (1)$$

If the assay procedure permits continuous monitoring of a signal, it is relatively easy to measure the time (t^*)

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required for a chosen amount of product (z^*) to be formed. This time is given by Eqn. 2.

$$t^* = z^*/V_m - (K_m/V_m) \cdot \ln(1 - z^*/A_0) \quad (2)$$

If t^* is measured for a series of A_0 then a plot of t^* vs. $-\ln(1 - z^*/A_0)$ will be a straight line with a slope of K_m/V_m and an intercept on the ordinate of z^*/V_m. From this slope and intercept, V_m and K_m are easily calculated. Alternatively, Eqn. 2 can be fitted to the data (z^*, t^*) by nonlinear regression to obtain values of V_m and K_m. In the results presented below, the graphical method is used for illustration although the data are analysed by nonlinear regression, using the DNRP53 computer program [8].

The fixed point which is recommended is half the lowest substrate concentration and this was chosen for the following reasons. If the fixed point is to be the same at all values of A_0, it clearly must be somewhat less than the lowest A_0. On the other hand, if it is experimentally possible for it to be very small there will be no real problem in determining v_0. Thus, z^* is likely to lie in the range from 10 to 90% of the lowest A_0, and 50% was taken to be a reasonable compromise.

Progress curves for pyruvate kinase were determined at 30 °C in 2.7 ml reaction mixtures containing 5.6 mM ADP, 11 mM $MgCl_2$, 22 mM KCl and 10 IU lactate dehydrogenase in 0.1 M Tris-HCl buffer (pH 8.0). The concentration of phospho*enol*pyruvate was varied (approx. 20–200 μM) and sufficient NADH added to ensure that there was an excess over phospho*enol*pyruvate of between 60 and 120 μM. The reaction was started by adding approx. 0.015 IU of pyruvate kinase and the absorbance at 340 nM was followed.

Application of the fixed-point method to the reaction catalysed by pyruvate kinase is illustrated in Fig. 1. These 22 experimental measurements were analysed by fitting Eqn. 2 to the data and gave $V_m = 9.08 \pm 0.31 \cdot 10^{-8}$ M s^{-1} and $K_m = 2.66 \pm 0.25 \cdot 10^{-5}$ M. This maximum velocity agrees well with that expected from the quantity of enzyme added (0.015 IU in 2.7 ml equals $9.26 \cdot 10^{-8}$ M s^{-1}), while the K_m for phospho*enol*pyruvate is similar to the value of $3.13 \cdot 10^{-5}$ M reported earlier [9].

One of the principal advantages of the fixed-point method over the half-time method [7] is that the latter requires long incubations, especially at high substrate concentrations. Indeed, if z^* is taken as half the lowest A_0, the longest incubation in the present method will require exactly the same time as the shortest one in the fixed-point method. Moreover, the half-time method requires accurate knowledge of A_0 which the authors suggest should be determined by allowing the reaction to run to completion, an even more time-consuming process. By contrast, it has been found by computer simulation (data not shown) that the present method is relatively insensitive to inaccuracies in A_0.

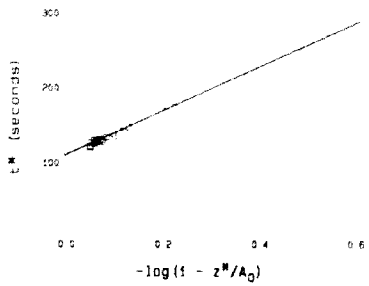

Fig. 1. Analysis of the pyruvate kinase reaction by the fixed-point method. Reactions were followed as described in the text and t^*, the time required to produce 10 μM NAD^+ (z^*) was determined over a range of A_0 (phospho*enol*pyruvate) values from 23 to 195 μM. Eqn. 2 was fitted to the data by nonlinear regression and the *line* represents this best fit.

While the method described here is targeted specifically at experiments to determine V_m and K_m, it can be used in other situations where initial rates would normally be measured. For example, when measuring the amount of an enzyme during its purification, one could compare $1/t^*$ since this value is proportional to v_0 at any given A_0.

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