ACTA CHEMICA SCANDINAVICA 23 (1969) 979–987

Kinetics of Enzymatic Formation of Products Submitted to First-Order Decomposition

GÖSTA PETTERSSON

Department of Biochemistry, University of Lund, Kemicentrum, Box 740, S-220 07 Lund 7, Sweden

The steady-state kinetics of the ordinary Michaelis-Menten case have been investigated under the assumption that the immediate enzymatic product is transformed into a final product by a non-enzymatic first-order reaction. The characteristic features of the functional interrelationship between time and product concentrations are described, and equations are derived which enable calculations of reaction rate constants and parameters from determinations of product concentrations as a function of time at different substrate concentrations. The general applicability of the proposed methods for parameter estimation is discussed, and the difficulty to distinguish between the kinetic properties of an enzyme-substrate complex and a very unstable intermediate product is emphasized.

In kinetic studies of the usual Michaelis-Menten case, where the conversion of a substrate S into a product B is enzymatically catalyzed in accordance with reaction (1), the reaction velocity can be measured by observing either the rate of disappearance of S or the rate of formation of B; under the steady-state assumption $-\mathrm{d[S]}/\mathrm{d}t$ equals $\mathrm{d[B]}/\mathrm{d}t$.[1] The latter relationship is not valid if B is unstable, *e.g.* if B participates in the non-enzymatic reaction (2) to give a final product P.

In relation to studies of enzyme systems where the reaction can be conveniently followed by observations of the concentration of an unstable product, it became necessary to undertake the present investigation on the characteristic kinetic properties of the general mechanism defined by reactions (1) and (2), and particularly to determine to what extent observations of [B] as a function of time t can be utilized for estimation of reaction rate constants and parameters such as the Michaelis-Menten constant.

THEORETICAL

Consider the mechanism defined by the reactions

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E+B \quad (1)$$

$$B \xrightarrow{k_3} P \quad (2)$$

The following elementary kinetic equations are obtained:

$$d[ES]/dt = k_1[S][E]-(k_{-1}+k_2)[ES] \quad (3)$$

$$d[S]/dt = -k_1[S][E]+k_{-1}[ES] \quad (4)$$

$$d[B]/dt = k_2[ES]-k_3[B] \quad (5)$$

$$c_E = [E]+[ES] \quad (6)$$

$$c_S = [S]+[ES]+[B]+[P] \quad (7)$$

where c_E and c_S stand for the total concentrations of enzyme and substrate, respectively. Assuming a quasi steady-state for the enzymatic reaction, *i.e.* putting $d[ES]/dt=0$, substitution of eqn. (6) in eqn. (3) gives [2]

$$[ES] = \frac{c_E[S]}{[S]+K} \quad (8)$$

where K stands for the quotient $(k_{-1}+k_2)/k_1$. From eqns. (4), (6), and (8) we get

$$-\frac{d[S]}{dt} = \frac{k_2 c_E[S]}{[S]+K} \quad (9)$$

and hence by integration [1]

$$k_2 c_E t = c_S-[S]-K \ln ([S]/c_S) \quad (10)$$

In the initial phase of the reaction $cs-[S]$ is small, and a series expansion of the logarithmic term in eqn. (10) with neglection of non-linear terms yields

$$t = \frac{(c_S-[S])(c_S+K)}{k_2 c_E c_S} \quad (11)$$

In the final phase we have $[S] \ll K$, and (still assuming a steady-state) the linear term in eqn. (10) may be neglected:

$$[S] = c_S \exp(-k_2 c_E t/K) \quad (12)$$

Under the steady-state assumption eqn. (5) becomes

$$\frac{d[B]}{dt} = \frac{k_2 c_E[S]-k_3[B]}{[S]+K} \quad (13)$$

This linear differential equation has the solution

$$[B] = k_2 c_E \exp(-k_3 t) \int_0^t [S]/([S]+K) \exp(k_3 t)\, dt \quad (14)$$

Change of variable using eqn. (9) yields

$$[B] = \exp(-k_3 t) \int_{[S]}^{c_S} \exp(k_3 t)\, d[S] \quad (15)$$

The integral in eqn. (15) can be exactly evaluated using eqn. (10) and a series expansion of $\exp(k_3 t)$, but this method yields [B] as a series expression in [S], which is of little practical value. For practical purposes an explicit expression of [B] as a function of t is desirable. This cannot, in general, be obtained, but examination of two extreme cases ($c_S \ll K$ and $c_S \gg K$) might illustrate the characteristics of the functional dependence of [B] on t for the general case.

For $c_S \ll K$ eqn. (12) is valid throughout the reaction, and observing that $[S]+K \approx K$ eqn. (14) becomes

$$[B] = k_2 c_E \exp(-k_3 t) \int_0^t (c_S/K) \exp(-k_2 c_E t/K) \exp(k_3 t)\, dt$$

whence

$$[B] = \frac{k_2 c_E c_S}{K k_3 - k_2 c_E} [\exp(-k_2 c_E t/K) - \exp(-k_3 t)] \quad (16)$$

or

$$[B] = c_S k_3 t \exp(-k_3 t) \quad (17)$$

where eqn. (16) is valid for $K k_3 \neq k_2 c_E$ and eqn. (17) for $K k_3 = k_2 c_E$. The [B]/t-curves obtained for different values of k_3 are shown in Fig. 1.

For $c_S \gg K$ the logarithmic term in eqn. (10) can be completely neglected, *i.e.* [S] decreases linearly with time

$$[S] = c_S - k_2 c_E t \quad (18)$$

approximately becoming zero at $t = c_S/k_2 c_E$. For $t < c_S/k_2 c_E$ we may use the approximation $[S]+K \approx [S]$ and eqn. (14) reduces to

$$[B] = k_2 c_E \exp(-k_3 t) \int_0^t \exp(k_3 t)\, dt$$

whence

$$[B] = \frac{k_2 c_E}{k_3} [1 - \exp(-k_3 t)] \quad (19)$$

For $t \geq c_S/k_2 c_E$ [S], and consequently [ES] (see eqn. (8)), may be put equal to zero and eqn. (5) reduces to

$$d[B]/dt = -k_3 [B] \quad (20)$$

It follows that [B] increases according to eqn. (19) in the early phase of the reaction, approaching a maximum value B_m which is obtained for $t=c_S/k_2c_E$; B_m may be calculated by insertion of $t=c_S/k_2c_E$ into eqn. (19). [B] then decreases by ordinary first-order kinetics according to eqn. (20), or in the integrated form

$$[B] = B_0 \exp(-k_3 t) \quad (21)$$

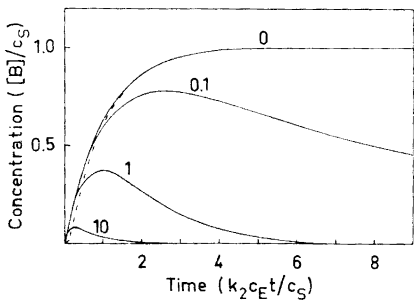

Fig. 1. [B]/t-curves for the case $c_S \ll K$, calculated from eqn. (16) for $k_3K/k_2c_E=0$, 0.1, 1, and 10. The dashed curve indicates [P] for $k_3K/k_2c_E=10$.

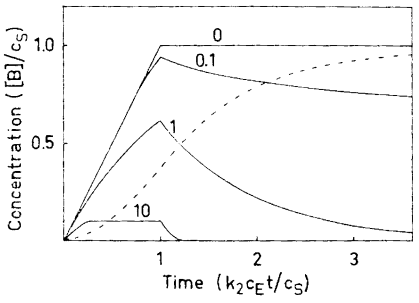

Fig. 2. [B]/t-curves for the case $c_S \gg K$, calculated from eqns. (19) and (21) for $k_3/k_2c_E=0$, 0.1, 1, and 10. The dashed curve indicates [P] for $k_3/k_2c_E=1$.

where t is counted from the moment when [B] equals B_0. The [B]/t-curves obtained for the case $c_S \gg K$ are shown in Fig. 2.

The case $c_S \ll K$ must be considered as fairly uncommon in steady-state kinetics. Usually, substrate concentrations are used which are much larger than or in the proximity of K. Fig. 3 shows the [B]/t-curves obtained for an intermediate case, where c_S is slightly larger than K.

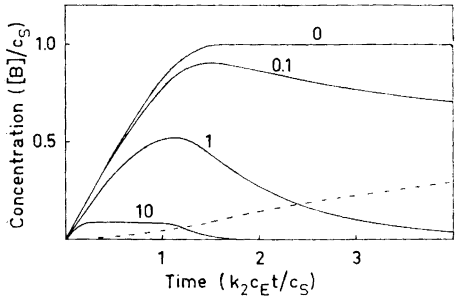

Fig. 3. [B]/t-curves for the case $c_S=10K=0.01$, calculated by standard numerical methods from eqns. (10) and (13) for $k_3/k_2c_E=0$, 0.1, 1, and 10. The dashed curve indicates [P] for $k_3/k_2ce=0.1$.

Inspection of eqns. (13)—(15) and Figs. 1—3 shows some general characteristics of the dependence of [B] on time:

1. [B] increases with time to a maximum value B_m at $t=t_m$, and then gradually decreases to zero.

2. B_m (t_m) approaches c_S (c_S/k_2c_E) as k_3 approaches zero.

3. When k_3 is small in comparison with $k_2c_Ec_S/(c_S+K)$, [B] is essentially determined by the enzymatic reaction (1) in the initial phase, and by the non-enzymatic reaction (2) in the final phase.

4. When k_3 is comparatively large B_m is rapidly reached, and [B] is generally maintained for some time at an approximately constant value.

One of the main problems in kinetic studies is to estimate reaction rate constants or parameters (such as K, k_2c_E, and k_3) from steady-state kinetic data, usually obtained using c_S as a controlled variable. Considering this question it will be essential to distinguish between two cases, depending on whether the non-enzymatic decay of B is rapid or not in comparison with the enzymatic reaction rate. Since the latter velocity can be altered by variation of c_E and/or c_S, it is always possible to carry out parameter determinations under one of the following two conditions:

I. Ordinary case: $k_3(c_S+K)/k_2c_Ec_S<1$

Initially (t small, although sufficiently large to fulfill the assumption of steady-state with respect to ES), the relationship between [S] and t is given by eqn. (11). Substituting this in eqn. (15)

$$[B] = \exp(-k_3t) \int_{[S]}^{c_S} \frac{(k_3(c_S-[S])(c_S+K)]}{k_2c_Ec_S} \, d[S]$$

whence

$$[B] = \frac{k_2c_Ec_S}{k_3(c_S+K)} [1-\exp(-k_3t)] \tag{22}$$

For small values of k_3t one may use the approximation

$$\exp(-k_3t) = 1-k_3t \tag{23}$$

and eqn. (22) reduces to

$$\frac{[B]}{t} = \frac{k_2c_Ec_S}{c_S+K} \tag{24}$$

This is the ordinary Michaelis-Menten equation, and determinations of the reaction rate (measured as the initial rate of formation of B) at different values of c_S can be used for estimation of K and k_2c_E by either statistical [3] or graphical [4] methods.

In the late phase of the reaction [ES] is negligibly small and eqn. (5) reduces to eqn. (20); [B] is given by eqn. (21), t being counted from an arbitrarily chosen moment when $[B]=B_0$. The rate constant k_3 can thus be determined from the final part of any observed [B]/t-curve, either statistically using eqn. (21) or graphically by plotting log[B] against t.

Fig. 4 shows the effect of variation of c_S for a typical case where the rate parameters may be determined as described above; arrows in the figure indicate times after which the decrease of [B] follows first-order kinetics. This first-

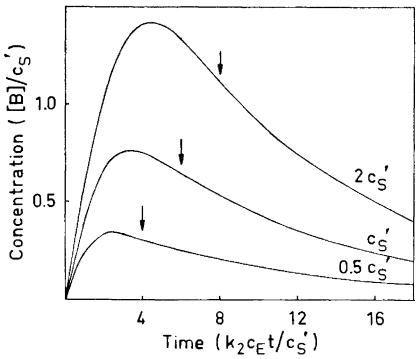

Fig. 4. Effect of variation of c_S on the [B]/t-curve for the case $c_S'=K$. Arrows indicate the time after which [B] decreases in accordance with eqn. (21). $k_3=0.1\ k_2c_E/K$.

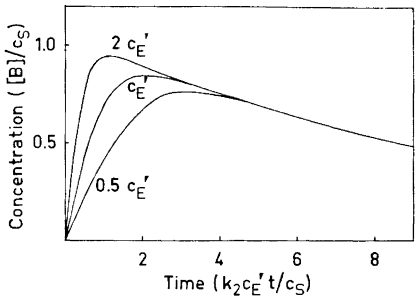

Fig. 5. Effect of variation of c_E on the [B]/t-curve for the case $c_S=K$ (*cf.* Ref. 5). $k_3=0.1\ k_2c_E/K$.

order phase can, evidently, be extended by increasing c_E to make the enzymatic transformation of S into B more rapid. Fig. 5 shows the effect of variation of c_E on the shape of the [B]/t-curves for a typical case.

II. Steady-state case: $k_3(c_S+K)/k_2c_Ec_S>10$

In this case d[B]/dt rapidly becomes zero, and remains small throughout the larger part of the reaction; [B] reaches a maximum value B_m which is essentially maintained until [S] becomes small (*cf.* Figs. 2 and 3). This means that a quasi steady-state approximation can be applied to B as well as to ES. Thus assuming that d[B]/dt=0 eqn. (5) becomes

$$[B] = B_m = (k_2/k_3)[ES] \tag{25}$$

Consequently, the variation of [B] merely reflects the variation of the steady-state concentration of ES.

Initially [S]=c_S and substitution of eqn. (8) in eqn. (25) gives (*cf.* eqns. (19) and (22) where exp(−k_3t) rapidly becomes negligible when k_3 is large)

$$B_m = \frac{k_2c_E}{k_3} \cdot \frac{c_S}{c_S+K} \tag{26}$$

The parameters K and k_2c_E/k_3 can thus be estimated from determinations of B_m at different values of c_S, either statistically using eqn. (26) or graphically by plotting $1/B_m$ against $1/c_S$.

In the final phase of the reaction [ES]≈c_E[S]/K, where [S] is given by eqn. (12). Substituting this in eqn. (25)

$$[B] = \frac{k_2c_Ec_S}{k_3K} \exp\left(\frac{-k_2c_Et}{K}\right) \tag{27}$$

which shows that the decrease of [B] appears to follow first-order kinetics with the apparent decay constant k_2c_E/K (instead of k_3 for the ordinary case; the two cases can obviously be distinguished by studying the effect of variation of c_E on the first-order constant). The parameter k_2c_E/K can be experimentally determined analogous to k_3 in the ordinary case, and combination with the initial data yields K, k_2c_E, and k_3.

The concentration of the final product P is determined by eqn. (7), and hence for $c_S \gg c_E$ by the simple relationship

$$[P] = c_S - [S] - [B] \qquad (28)$$

It follows that [P] steadily increases with time, and typical shapes of the [P]/t-curves obtained (calculated using eqns. (10) and (28)) are indicated in Figs. 1—3 (dashed curves).

In the ordinary case (k_3 comparatively small) the functional relationship between [P] and t is fairly complicated in the initial reaction phase, and cannot easily be used for experimental parameter evaluation. In the final phase, however, the increase of [P] reflects the decrease of [B] according to eqn. (21), and the corresponding part of the [P]/t-curve can be used for determination of k_3, *e.g.* graphically by plotting $\log(c_S - [P])$ against t.

In the steady-state case (k_3 comparatively large) we have $d[B]/dt = d[ES]/dt = 0$, and derivation of eqn. (7) yields $d[P]/dt = -d[S]/dt$. This means that P kinetically may be regarded as the immediate enzymatic product (*cf.* Fig. 1, where the dashed [P]/t-curve obtained for $k_3 = 10k_2c_E/K$ closely agrees with the [B]/t-curve for $k_3 = 0$), and determinations of the rate of formation of P can be used for estimation of the parameters K and k_2c_E in the same manner as described for the usual Michaelis-Menten case.[4]

DISCUSSION

Several enzymatically catalyzed reactions have been reported to give rise to unstable products. Most of these reports concern systems where the decay of the immediate product is extremely slow in comparison with the enzymatic reaction rate. This can be examplified by the ceruloplasmin (EC 1.12.3) catalyzed oxidation of *N*,*N*-dimethyl-*p*-phenylenediamine to Wurster's red, where the enzymatic part of the process is completed within some minutes, while the half-time for the non-enzymatic decay of Wurster's red is in the order of 10—20 h.[5] For such systems it is evident that an ordinary kinetic treatment (assuming formation of a stable enzymatic product) can be applied to the enzymatic part of the reaction.

A less extreme case can be illustrated by the example of 3-hydroxyanthranilate oxygenase (EC 1.99.2), where the final formation of quinolinic acid has been found to proceed *via* 2-amino-3-carboxymuconic acid semialdehyde. The decay constant k_3 for this intermediate has been obtained by application of eqn. (21) to the final reaction phase, and these calculations are justified by the fact that denaturation of the enzyme has no effect on the k_3 values obtained.[6] Ogasawara *et al.* recently reported of the determination of rate parameters for the enzymatic part of the system.[7] In these studies,

which appear to assume formation of a stable product, the enzymatic reaction velocity was determined as the rate of formation of intermediate during the first 5—15 sec.

The results of the present investigation show that the enzymatic reaction rate can be identified with the initial value of d[B]/dt without assuming formation of a stable product B. Besides being dependent on the usual requirement that $c_S - [S]$ is small (which, in general, can be met by increasing c_S and/or decreasing c_E), the validity of eqn. (24) is only based upon the assumption that eqn. (23) is valid, which is fulfilled on the 1 % level of error for $t < 0.02/k_3$. In the case of 3-hydroxyanthranilate oxygenase ($k_3 = 0.014$ min^{-1}) initial studies may be carried out for at least 2 min.

For k_3 values larger than 0.1 min^{-1} initial velocity determinations have to be performed within the first few seconds of the reaction, also when k_3 is comparatively small. It may then be advantageous to decrease the enzymatic reaction rate (which can be done by decreasing c_E and/or c_S) in order to obtain a steady-state concentration of B, and to carry out determinations of rate parameters using eqns. (26) and (27). The application of this steady-state method for parameter evaluation is dependent upon the assumption that $\exp(-k_3 t)$ becomes negligible while $c_S - [S]$ is still small. It can easily be shown that (on the 1 % level of error) these requirements imply that $B_m > 0.004 c_S$. Hence it follows that the enzymatic reaction rate preferably should be modified by decreasing c_E instead of c_S.

While low c_E values usually are preferable in initial studies determinations of k_3 from the final reaction phase are facilitated by using high concentrations of enzyme. Besides extending the time during which eqn. (21) is applicable, increase of c_E gives a general higher concentration of the intermediate product (see Fig. 5).

No reports on enzyme systems yielding unstable products seem to have been concerned with the case of comparatively large k_3 values. This is not astonishing as enzymatic reactions, in general, are rapid in comparison with non-enzymatic processes. Furthermore, it follows from eqn. (26) that the steady-state concentration B_m is less than $k_2 c_E / k_3$. A large k_3 value implies that the intermediate product is kept at a very low and approximately constant concentration throughout the reaction. Under such circumstances it seems unlikely that attention is drawn to the presence of an intermediate product; the final product P will probably be regarded as a primary enzymatic product, (*cf.* [P]/t-curve in Fig. 1).

The requirement $B_m > 0.004 c_S$ (see above) appears to limit the practical applicability of the steady-state method for parameter evaluation; when substrate concentrations in the order of 1 mM are used, accurate methods must be available for determination of B_m on the 1 μM level. Theoretically, however, the steady-state case is of great importance, as several enzymatic mechanisms may include the formation of very unstable intermediate products (*e.g.* radicals, or compounds that react rapidly with water or oxygen in a pseudo first-order reaction). Such unstable compounds might show strong electromagnetic absorption, and might thus be detected also when present in small amounts. Since the concentration of such an intermediate will be proportional to the concentration of enzyme-substrate complex (see eqn. (25)),

it could be very difficult to distinguish kinetically between these participants of the reaction and there is always a potential risk of false interpretation of kinetical data which are postulated to concern the enzyme-substrate complex. The attribution of certain observed (*e.g.* spectral) properties of an enzyme system to the enzyme-substrate complex must always be tested against the possibility that these properties are due to the presence of small steady-state amounts of an unstable intermediate product. It is uncertain whether this possibility, hitherto, has been properly regarded.

The above results concerning the mechanism defined by the reactions (1) and (2) can directly be generalized to cover various types of mechanisms showing the following basic properties:

a. The enzymatic reaction yields an unstable product B in one single, irreversible, step.

b. The decay of B follows first-order kinetics.

c. The enzymatic reaction velocity depends hyperbolically on substrate concentration, *i.e.* the rate equation is of the Michaelis-Menten type with respect to substrate if the product is assumed to be stable.

This means, for instance, that reaction (1) may be exchanged for a sequence of reactions involving several enzymatic complexes C_i, as indicated in (29),

$$E+S \rightleftharpoons C_1 \rightleftharpoons C_2 \rightleftharpoons \cdots \rightleftharpoons C_n \longrightarrow E+B \qquad (29)$$

and that ordinary studies on the effect of modifiers may be performed using the above methods for parameter evaluation. For such extended mechanisms the interpretation of the parameter values K and k_2c_E must be modified in analogy to the necessary modifications for the corresponding cases with formation of a stable product.[8]

The extension of the present theory to systems which do not obey Michaelis-Menten kinetics with respect to the substrate concentration (*e.g.* systems showing substrate inhibition) is straight forward and will not be discussed.

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Received June 18, 1968.