THE KINETIC ANALYSIS OF HYDROLYTIC ENZYME CATALYSES: CONSEQUENCES OF NON-PRODUCTIVE BINDING

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Received 30 September 1968

Evidence has accumulated during the last few years which strongly supports the description of the reactions catalysed by a number of hydrolytic enzymes in terms of the three step mechanism, eq. (1), involving the intermediacy of an enzyme-substrate complex (ES) and an acyl-enzyme (ES') [1–8]. One of the enzymes

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\,P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

for which the evidence in favour of this type of kinetic mechanism is particularly convincing is papain (EC 3.4.4.10). Thus Lowe and Williams [8] convincingly demonstrated that the papain-catalysed hydrolysis of methyl thionohippurate involves the formation of an acyl-enzyme intermediate in which the acyl moiety of the substrate is linked to the active centre cysteine residue by a thiol ester bond. One of the major problems in the study of enzyme catalyses described by eq. (1) is the isolation of values for the first order rate constants for both acylation (k_2) and deacylation (k_3) or at least the assessment of their relative magnitudes. The recent approaches to the solution of this problem by Whitaker and Bender [9] and by Sluyterman [10] yield conflicting results when applied to papain-catalysed hydrolyses. Whitaker and Bender determined k_2 and k_3 separately for the papain-catalysed hydrolysis of BAEE * and found that k_2/k_3 is ca. 3, i.e. deacylation is marginally rate-limiting. Sluyterman, however, concluded from his analysis that for the BAEE hydrolysis $k_2 \ll k_3$, i.e. acylation is rate-limiting. In an attempt to interpret the kinetics of bromelain-catalysed hydrolyses, we recently applied the analysis devised by Whitaker and Bender and suggested that the binding of BAA to bromelain may involve a strong non-productive component [11]. This together with the possibility that non-productive binding may be generally a significant feature in enzyme-substrate interaction (e.g. [12]) led us to consider the effect of non-productive binding on the kinetic analyses devised by Whitaker and Bender and by Sluyterman to ascertain whether this could account for their apparent incompatibility.

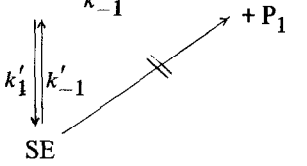
We report that if the enzyme binds the substrate not only in such a way that acylation occurs but also in a mode that will not permit acylation (non-productive binding) the analysis of the k_{cat}–K_m(app) data by a method analogous to that of Whitaker and Bender yields the correct value for k_3 but a low estimate of k_2. Thus non-productive binding would serve only to enhance the degree to which deacylation appears from the analysis of Whitaker and Bender to be rate-limiting in the papain-BAEE system. We report also that the Sluyterman analysis when modified to take account of non-productive binding does not now allow assessment of the relative magnitudes of k_2 and k_3. This extended treatment demonstrates that Sluyterman's findings that whereas BGEE protects papain from alkylation by CAA, BAEE offers no protection, may be explained without contradicting the results of the Whitaker and Bender analysis if BAEE binds to papain in a non-productive mode.

* Abbreviations used: BAA, α-N-benzoyl-L-arginine amide; BAEE, α-N-benzoyl-L-arginine ethyl ester; BGEE, benzoylglycine ethyl ester; CA, chloroacetamide; CAA, chloroacetic acid; IA, iodoacetamide.

Modification of the Whitaker and Bender analysis

Extension of the acyl-enzyme mechanism, eq. (1), to include the binding of the substrate by the enzyme in modes which will not permit acylation may be represented by eq. (2) in which SE is the enzyme-substrate complex or complexes which cannot give rise to the acyl-enzyme ES'.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (2)$$



Application of the steady state treatment to eq. (2) yields the rate equation (3), in which the various parameters are defined by eqs. (4)–(6).

$$\frac{dP_2}{dt} = \frac{dP_1}{dt} = \frac{\{k_2k_3K'_s/(k_3K'_m + k_3K'_s + k_2K'_s)\}[E_T][S]}{[S] + k_3K'_sK_m/(k_3K_m + k_3K'_s + k_2K'_s)} \qquad (3)$$

$$[E_T] = [E] + [ES] + [SE] + [ES'] \qquad (4)$$

$$K_m = (k_{-1} + k_2)/k_1 \qquad (5)$$

$$K'_s = k'_{-1}/k'_1 . \qquad (6)$$

The constants of eq. (3) are related to those of the usual Michaelis-Menten equation (7) by eqs. (8) and (9).

$$E + S \underset{}{\overset{K_m(app)}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (7)$$

$$k_{cat} = k_2k_3/\{(k_3K_m/K'_s) + k_2 + k_3\} \qquad (8)$$

$$K_m(app) = k_3K_m/\{(k_3K_m/K'_s) + k_2 + k_3\} \qquad (9)$$

Equations analogous to eqs. (8) and (9) have been derived previously [13] for the two-step kinetic mechanism, eq. (7). Comparison of eqs. (8) and (9) with the analogous eqs. (10) and (11) which result from the steady state treatment of eq. (1) demonstrates that non-productive binding would result in low experimental estimates of both k_{cat} and K_m(app) but that the ratio k_{cat}/K_m(app) would be unaffected, where

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (10)$$

and

$$K_m(app) = k_3K_m/(k_2 + k_3) . \qquad (11)$$

When non-productive binding may be neglected i.e. $K'_s \to \infty$, eqs. (8) and (9) reduce to eqs. (10) and (11) respectively. If it is assumed that $k_{-1} \gg k_2$, eqs. (8), (9) and (11) become eqs. (12), (13) and (14), respectively, in which K_s is the dissociation constant of ES, i.e. k_{-1}/k_1.

$$k_{cat} = k_2k_3/\{(k_3K_s/K'_s) + k_2 + k_3\} \qquad (12)$$

$$K_m(app) = k_3K_s/\{(k_3K_s/K'_s) + k_2 + k_3\} \qquad (13)$$

$$K_m(app) = k_3K_s/\{(k_2 + k_3)\} \qquad (14)$$

Eq. (15) follows both from eqs. (12) and (13) and from eqs. (10) and (14).

$$k_{cat}/K_m(app) = k_2/K_s . \qquad (15)$$

If k_2 is eliminated from eqs. (13) and (15), eq. (16) results:

$$k_{cat} = k_3 - k_3K_m(app)(K_s + K'_s)/K_sK'_s . \qquad (16)$$

This may be compared with the Whitaker and Bender equation (17) for the system in which non-productive binding is neglected, which results from elimination of k_2 from eqs. (14) and (15).

$$k_{cat} = k_3 - k_3K_m(app)/K_s . \qquad (17)$$

If k_3 and K_s are independent of pH, eq. (17) predicts that a plot of k_{cat} against K_m(app) for data obtained at various pH's will be linear with intercepts of k_3 and K_s on the k_{cat} and K_m(app) axes respectively. If the same data were analysed using eq. (16) which takes account of the possibility of non-productive binding, the intercept on the k_{cat} axis would still be k_3 but the intercept on the K_m(app) axis would be $K_sK'_s/(K_s + K'_s)$ instead of simply K_s. Thus although this method of analysis provides the correct value for k_3 it provides a low estimate of K_s if non-productive binding is of significance and hence a low estimate of k_2 which is obtained by substitution of K_s into eq. (15).

Modification of the Sluyterman Analysis

The Sluyterman method of determining whether acylation or deacylation is rate-limiting in a given papain catalysed hydrolysis consists in the measurement of the rate of inactivation of papain by alkylation with CAA of the active centre thiol group, in the presence of various concentrations of substrate.

According to eq. (1), total active enzyme is present in three states E, ES and ES′. In ES′ the thiol group is linked covalently to the acyl moiety of the substrate and therefore cannot undergo alkylation by CAA. The rate of inactivation may be represented, therefore, by eq. (18) in which k_f and k_c are the inactivation rate constants of E and ES respectively and k is the experimentally determined pseudo first order rate constant for the inactivation divided by the concentration of CAA.

$$\text{Rate} = k[E_T] = k_f[E] + k_c[ES] \ . \qquad (18)$$

Eqs. (19) and (20) which result from the steady state treatment of eq. (1), the conservation eq. (21), and eq. (18) may be combined to yield eq. (22).

$$[ES'] = \{k_2/k_3\}[ES] \qquad (19)$$

$$[E] = [E_T]K_m(\text{app})/\{[S] + K_m(\text{app})\} \qquad (20)$$

$$[E_T] = [E] + [ES] + [ES'] \qquad (21)$$

$$k = \left\{k_f - \frac{k_c k_3}{k_2+k_3}\right\} \frac{K_m(\text{app})}{[S] + K_m(\text{app})} + \frac{k_c k_3}{k_2+k_3} \qquad (22)$$

In eqs. (20)–(22) K_m(app) is defined for eq. (1) by eq. (11). Eq. (22) predicts that a plot of k against $K_m(\text{app})/\{[S] + K_m(\text{app})\}$ will be linear. This was observed when both BAEE and BGEE were used as substrates. It follows from eq. (22) that when $[S] = 0$, k is given by eq. (23) and when $[S] = \infty$, k is given by eq. (24).

$$k = k_f \qquad (23)$$

$$k = k_c k_3/(k_2 + k_3) \ . \qquad (24)$$

When BGEE is used as substrate the straight line obtained in the plot of k against $K_m(\text{app})/\{[S] + K_m(\text{app})\}$ extrapolates at $[S]=0$ to a value of k_f which is closely similar to that measured in the absence of substrate. The extrapolation of the plot to

$$K_m(\text{app})/\{[S] + K_m(\text{app})\} = 0,$$

i.e. $[S] = \infty$, yields $k_c k_3/(k_2 + k_3) = 0$. From this result Sluyterman concluded that $k_2 \gg k_3$ for the BGEE hydrolysis. He rejected the alternative possibility, that $k_c = 0$, because his interpretation of his analysis for the hydrolysis of the larger substrate BAEE (see below) provides a value for k_c for this system which is equal to k_f.

For the BAEE hydrolysis the plot of k against $K_m(\text{app})/\{[S] + K_m(\text{app})\}$ gives a straight line parallel to the $K_m(\text{app})/\{[S] + K_m(\text{app})\}$ axis and the mean value of k is closely similar to that of k_f measured in the absence of substrate. Because the line is horizontal, extrapolation to zero and infinite substrate concentrations yield equal values of k, i.e., $k_f = k_c k_3/(k_2 + k_3)$. Sluyterman rejected a fortuitous compensation of k_c by $k_3/(k_2 + k_3)$ which would make the whole expression equal to k_f and concluded instead that $k_c = k_f$ which implies that $k_2 \ll k_3$.

The result of this analysis i.e. $k_c = k_f$ seems to us to be most unlikely if ES represents an enzyme-substrate complex which leads to acylation as shown in eq. (1). Since BAEE is a trifunctional substrate, binding of the benzoylamino group and the guanidine side chain to the ρ_1 and ρ_2 sites of papain will result in the alignment of the carbethoxy group of BAEE with the ρ_3 site which contains the reactive thiol group. Thus the reactivity of this thiol group towards CAA when BAEE is bound with its carbethoxy group in close proximity to it (reflected in k_c) would be expected to be considerably different (probably much lower) from that of the thiol group of the free enzyme (reflected in k_f), i.e. as with BGEE. This great difficulty would not arise if BAEE were bound to papain also in a non-productive mode, i.e. in such a way that no large part of the BAEE molecule were aligned with the reactive thiol group of the enzyme.

The consequences which non-productive binding would have on the significance of the Sluyterman analysis may be determined by deriving an equation analogous to eq. (22) using as a basis eq. (2) instead of eq. (1). The rate of inactivation previously given by eq. (18) is now given by eq. (25) in which k_d is the inactivation rate constant of the non-productive complex or complexes (SE).

$$\text{Rate} = k[E_T] = k_f[E] + k_c[ES] + k_d[SE] \ . \qquad (25)$$

Eqs. (26) and (19) which result from the steady state treatment of eq. (2), the conservation equation, (4), and eq. (25) may be combined to yield eq. (27). Eq. (26) takes the same form as eq. (20) but in eqs. (26) and (27), K_m(app) is defined for eq. (2) by eq. (28) and not by eq. (11).

$$[E] = [E_T]K_m(\text{app})/\{[S] + K_m(\text{app})\} \qquad (26)$$

$$k = \left\{k_f - \frac{k_c k_3}{k_2+k_3} - \left(\frac{k_c k_3}{k_2+k_3}\right)\frac{[S]}{K'_s} + \frac{k_d[S]}{K'_s}\right\} \times \frac{K_m(\text{app})}{[S]+K_m(\text{app})} + \frac{k_c k_3}{k_2+k_3} \qquad (27)$$

$$K_m(\text{app}) = k_3 K_m K'_s/(k_2 K'_s + k_3 K'_s + k_3 K_m) \qquad (28)$$

Eq. (27) predicts, as does eq. (22), that extrapolation to $[S] = 0$ will provide a rate of inactivation identical with that measured in the absence of substrate, i.e., that given by eq. (23). The difference between eqs. (22) and (27) lies in the expression for the rate of inactivation obtained by extrapolation to $[S] = \infty$. If $[S] \gg K_m$(app), eq. (27) becomes eq. (29) and when $[S] = \infty$ this becomes eq. (30) which gives the value of k obtained by extrapolation of $[S]$ to ∞.

$$k = K_m(\text{app})\left\{\frac{k_f}{[S]} - \left(\frac{k_c k_3}{k_2+k_3}\right)\frac{1}{[S]} - \left(\frac{k_c k_3}{k_2+k_3}\right)\frac{1}{K'_s} + \frac{k_d}{K'_s}\right\} + \frac{k_c k_3}{k_2+k_3} \qquad (29)$$

$$k = \frac{k_d K_m(\text{app})}{K'_s} + \frac{k_c k_3}{k_2+k_3}\left\{1 - \frac{K_m(\text{app})}{K'_s}\right\}. \qquad (30)$$

Eq. (30) may be compared with eq. (24) which gives the value of k obtained by extrapolation of $[S]$ to ∞ when non-productive binding is not considered.

When non-productive binding may be neglected, i.e. $K'_s \to \infty$, eq. (30) reduces to eq. (24). If non-productive binding features in the interaction of enzyme and substrate, however, eq. (30) does not readily allow an estimation of the relative magnitudes of k_2 and k_3.

We have shown that non-productive binding of BAEE by papain would not affect the value of k_3 obtained by the method of Whitaker and Bender but would result in a low estimate of k_2 obtained by this method. We would like to propose an alternative interpretation of Sluyterman's data in terms of eq. (30) which is not in conflict with the result of the Whitaker and Bender analysis of the papain-catalysed hydrolysis of BAEE.

When BAEE binds to papain in a productive mode, i.e. in such a way that acylation of the reactive thiol group of papain occurs, it seems likely that the carbethoxy group of the substrate would protect the thiol group from alkylation by CAA, i.e. $k_c \ll k_f$. If $k_c = 0$, eq. (30) becomes eq. (31).

$$k = k_d K_m(\text{app})/K'_s \; . \qquad (31)$$

If this condition obtains, the inactivation at infinite substrate concentration consists entirely of reaction of CAA with papain carrying substrate bound in a non-productive mode. Since the rate of inactivation at infinite substrate concentration is equal to k_f, eq. (31) becomes eq. (32).

$$k_f = k_d K_m(\text{app})/K'_s \; . \qquad (32)$$

Since it can be shown from eq. (28) that K'_s must be greater than or equal to K_m(app), the implication of eq. (32) is that k_d must be greater than or equal to k_f. If $k_d \doteq k_f$, the non-productive binding of BAEE to the enzyme does not change significantly the reactivity of the thiol group towards CAA. If this is the case, $K'_s \doteq K_m$(app) i.e. the binding of BAEE is very largely non-productive. If $k_d > k_f$ by the ratio K'_s/K_m(app), the non-productive binding of BAEE has resulted in an increase in the reactivity of the papain thiol group. It is of interest in this connection that the reactivity towards alkylation by CA and IA of the reactive thiol group of the closely related enzyme ficin (EC 3.4.4.12) was found to be increased by the presence of substrates and products [14]. Such an effect is possible if the binding results in a change in the nature of the environment of the thiol group by conformational changes. Eq. (30) shows that if $K'_s = K_m$(app), it is not necessary to assume that $k_c = 0$ to obtain the result that $k\,(= k_f) = k_d$.

In the case of the papain-BGEE system, we suggest that non-productive binding as defined above does not contribute greatly to the binding of this

substrate by papain. Whereas BAEE is a trifunctional substrate, BGEE is only bifunctional which means that even "wrong-way binding" can be productive in the sense that it can sterically align the carbethoxy group with the thiol group of the enzyme. We have shown above that when non-productive binding may be neglected, eq. (30) reduces to eq. (24). We suggest that the full protection which BGEE affords papain towards alkylation by CAA is explained most readily in terms of eq. (24) if it is assumed that $k_c=0$. In this case, this method of analysis provides no information about the relative magnitudes of k_2 and k_3. Theoretically there is no need to make the assumption that binding of BGEE does not involve a substantial non-productive component, if $k_d \doteq 0$. Since the above discussion assigns a non-zero value to k_d for the papain-BAEE system, however, it seems unlikely that k_d for the papain-BGEE system would be zero or near zero.

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