ON THE MECHANISM OF THE α-CHYMOTRYPSIN-CATALYSED HYDROLYSIS OF 4-*CIS*-BENZYLIDENE-2-PHENYLOXAZOLIN-5-ONE: EVIDENCE FOR COVALENT NON-PRODUCTIVE BINDING

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Discussion

During recent years the use of 4-*cis*-benzylidene-2-phenyloxazolin-5-one * (CBPO) and its derived methyl ester, methyl α-benzamido-*cis*-cinnamate, as substrates for α-chymotrypsin has been the subject of a number of papers both from this laboratory [1–4] and from that of Zerner [5,6]. The purpose of this communication is to point out apparent inconsistencies in the results reported from the two laboratories, to confirm both sets of results, and to propose an interpretation which both rationalises the two sets of data and suggests interesting mechanistic consequences of the apparent inconsistencies.

To initiate studies on the α-chymotryptic hydrolysis of substrates which possess both an *N*-acylamino side chain and the chromophoric cinnamoyl moiety, we measured the rate of hydrolysis catalysed by α-chymotrypsin of methyl α-benzamido-*cis*-cinnamate. The value of k_{cat} at 25.0° in phosphate buffer, I = 0.1, pH 7.9 containing 4.8% v/v dioxan was found to be 0.1 sec^{-1} [1]. Although this value may be subject to error because of the low concentrations of substrate which its limited solubility neccessitated, we regard this as a low estimate of k_3, the rate constant for the deacylation of the acyl-enzyme intermediate, α-benzamido-*cis*-cinnamoyl-α-chymotrypsin, detected spectrophotometrically in this catalysis [1]. We base this view on the fact that k_{cat} for this catalysis may be the well-known assembly of rate constants $k_2k_3/(k_2 + k_3)$ where k_2 is the acylation rate constant, which will always be less than either k_2 or k_3, and also the indication that this catalysis may be subject to substrate inhibition.

We next measured k_3 for the deacylation of α-benzamido-*cis*-cinnamoyl-α-chymotrypsin directly by following the first-order decay at 310 nm of the absorption band of the acyl-enzyme generated by allowing an excess of α-chymotrypsin to react with the acylating agent CBPO [2]. We found that at 25.0° in acetate and phosphate buffers, I = 0.1, containing 4.8% v/v dioxan in the pH range ca. 5.5–7.0, the deacylation is dependent upon a single ionizing group required for activity in the base form i.e. $k_3 = \bar{k}_3/(1 + [H^+]/K)$ where $\bar{k}_3$ = 0.13 sec^{-1} and pK = 7.68. In the pH range ca. 7–9, however, the observed rate constants were found to be much less than those predicted by the above rate equation and this was tentatively interpreted in terms of an N → O acyl shift. The experiments in the higher pH range were carried out in tris buffer and we subsequently reported [3] that when the deacylation experiments in the pH range 7–10 are carried out in phosphate and borate buffers the rate of deacylation follows a

* Although the absolute configuration of the geometrical isomers of 4-benzylidene-2-phenyloxazolin-5-one and their derived esters and acyl-enzymes have not been definitely established, the stable oxazolinone used in this work is assumed to possess the *cis*-configuration. For a discussion of the configuration of these compounds see Buckles et al. [13], Stefanovic and Stefanovic [14], Kochetkov et al. [15], Filler [16], Brocklehurst, Price and Williamson [4], Morgenstern et al. [17] and de Jersey and Zerner [6]. A final decision awaits the determination of the structure of the structure of these compounds by X-ray crystallography which is in progress.

rate equation of the above form where $\bar{k}_3 = 0.154$ sec^{-1} and $pK = 7.64$. We suggested [3] that the anomalous results obtained in tris buffer might be due to acylation of tris by CBPO. De Jersey and Zerner subsequently confirmed this suggestion and reported more extensive studies of this anomalous tris reaction [5,6]. These authors reported at the same time the following Michaelis parameters for the α-chymotryptic hydrolysis of CBPO in 0.1 M phosphate buffer, pH 7.93 containing 10% v/v acetonitrile: $k_{cat} = 0.033$ sec^{-1}; K_m(app) = 4×10^{-7} M. These parameters were obtained by conventional initial velocity measurements using $[E] \ll [S_o]$ and the loss of ultraviolet absorption in the region 360–380 nm as a measure of the disappearance of substrate. de Jersey and Zerner [6] regard their value of K_{cat} (0.033 sec^{-1}) as the value of k_3 for the catalysis. They appear to have overlooked the values of k_3 which we reported both in the paper [2] to which they refer in connection with our tentative suggestion involving N → O acyl transfer and also over a more extensive pH range in our subsequent paper [3] where we reported for the first time the anomalous tris reaction to which these authors [6] do not refer. These values of k_3, measured directly, were obtained in buffers containing 4.8% v/v dioxan. We have repeated these measurements at several pH's using buffers containing 10% v/v acetonitrile and have obtained closely similar (slightly higher) values. Using $[S_o] = 10^{-5}$ M, the same value of k_3 at a given pH is obtained using different enzyme concentrations in the range $5 \times 10^{-5} - 5 \times 10^{-4}$ M. We had previously measured the initial velocities under Michaelis-Menten conditions of the catalysis of the hydrolysis of CBPO by α-chymotrypsin by following the loss of ultraviolet absorption at 360 nm using a range of substrate concentrations all of which are much greater than the value of K_m(app) (4×10^{-7} M) for the catalysis reported by de Jersey and Zerner. These values of the initial velocity divided in each case by the enzyme concentration are here presented as values of k_{cat} in table 1. The average value of k_{cat} (0.037 sec^{-1} at pH 8.0) is in good agreement with that reported by de Jersey and Zerner (0.033 sec^{-1} at pH 7.93). The scatter on the results is probably the result of using such a low concentration of α-chymotrypsin which leads to plating out of the protein on the spectroscopic cells and volumetric glassware.

Table 1
Zero-order kinetics of the α-chymotrypsin-catalysed hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one.

10^7 $[S_o]$, M	k_{cat}, sec^{-1}
93.2	0.036
65.0	0.038
55.9	0.029
46.6	0.044
37.3	0.033
27.9	0.042
	Average $k_{cat} = 0.037$ sec^{-1}

Solvent, phosphate buffer pH 8.0, $I = 0.1$, containing 10% v/v acetonitrile; 25.0°; $[E] = 3.16 \times 10^{-8}$ M; reaction followed at 360 nm.

Since the value of k_3 measured directly is 3–4 times greater than the value of k_{cat} for the α-chymotrypsin-catalysed hydrolysis of CBPO, k_{cat} cannot be a measure of k_3 for this catalysis as is claimed by de Jersey and Zerner [6].

If it is assumed that the usual 3-step mechanism Scheme (1) * describes the catalysis of the hydrolysis of CBPO by α-chymotrypsin, the obvious interpretation of the low value of k_{cat} relative to that of k_3 is that under zero order conditions, acylation is contributing to the rate of the catalysis; if $k_2 = k_3/2$, $k_{cat} = k_2k_3/(k_2 + k_3) = k_3/3$.

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

That this simple interpretation does not apply in this case is suggested by the observation of de Jersey and Zerner [6] that when $[S_o] \gg K_m$, there is an initial rapid decrease in absorbance (or burst) equivalent to a decrease in substrate concentration equal to that of the enzyme. In terms of the above 3-step mechanism this equivalence of the initial burst to the enzyme concentration implies that $k_2 \gg k_3$ [7]. The 3-step mechanism (1) therefore appears to be an inadequate model to explain both the fact that $k_{cat} < k_3$ and that there is an initial burst equivalent to the enzyme concentration.

* When the substrate is an oxazolinone, which may be regarded as an internal ester, there is no P_1.

To reconcile these findings it is here suggested that the catalysis of the hydrolysis of CBPO by α-chymotrypsin under Michaelis-Menten conditions is described by Scheme (2) which is the 3-step mechanism (1) extended to include the binding of the substrate in a non-productive mode, ES″. This is essentially the same kinetic scheme as that which we have discussed previously in another connection [8]. In Scheme (2) ES is the non-covalently bonded (Michaelis) complex of the substrate and

$$\begin{array}{ccccccc} & & k_1 & & k_2 & & k_3 \\ E + S & \rightleftharpoons & ES & \rightarrow & ES' & \rightarrow & E + P \\ & & k_{-1} & & & & \end{array} \qquad \text{Scheme (2)}$$

$$k'_{-1} \uparrow\downarrow k'_1$$

$$ES''$$

the enzyme whose absorption spectrum in the region of 360 nm should approximate that of the substrate itself. ES′ is the acyl-enzyme, α-benzamido-*cis*-cinnamoyl-α-chymotrypsin and ES″ is another covalent enzyme-substrate compound. To explain the quantitative initial burst it is a requirement that ES″ like ES′ should be a compound in which the conjugated system linking the two phenyl groups in CBPO which is responsible for the intense absorption band at 360 nm has been destroyed. The formation of the covalent compound ES″ may involve the intermediacy of a non-covalent (Michaelis) complex which is usually assumed to be formed in enzyme reactions although direct acylation without such an adsorptive intermediate has been suggested to explain the kinetics of the elastase-catalysed hydrolysis of *p*-nitrophenyl trimethyl acetate [9]. Although Scheme (2) is the simplest model for non-productive covalent binding, it is possible that ES″ breaks down to give eventually enzyme and product at a slower rate than the reaction which goes via ES′.

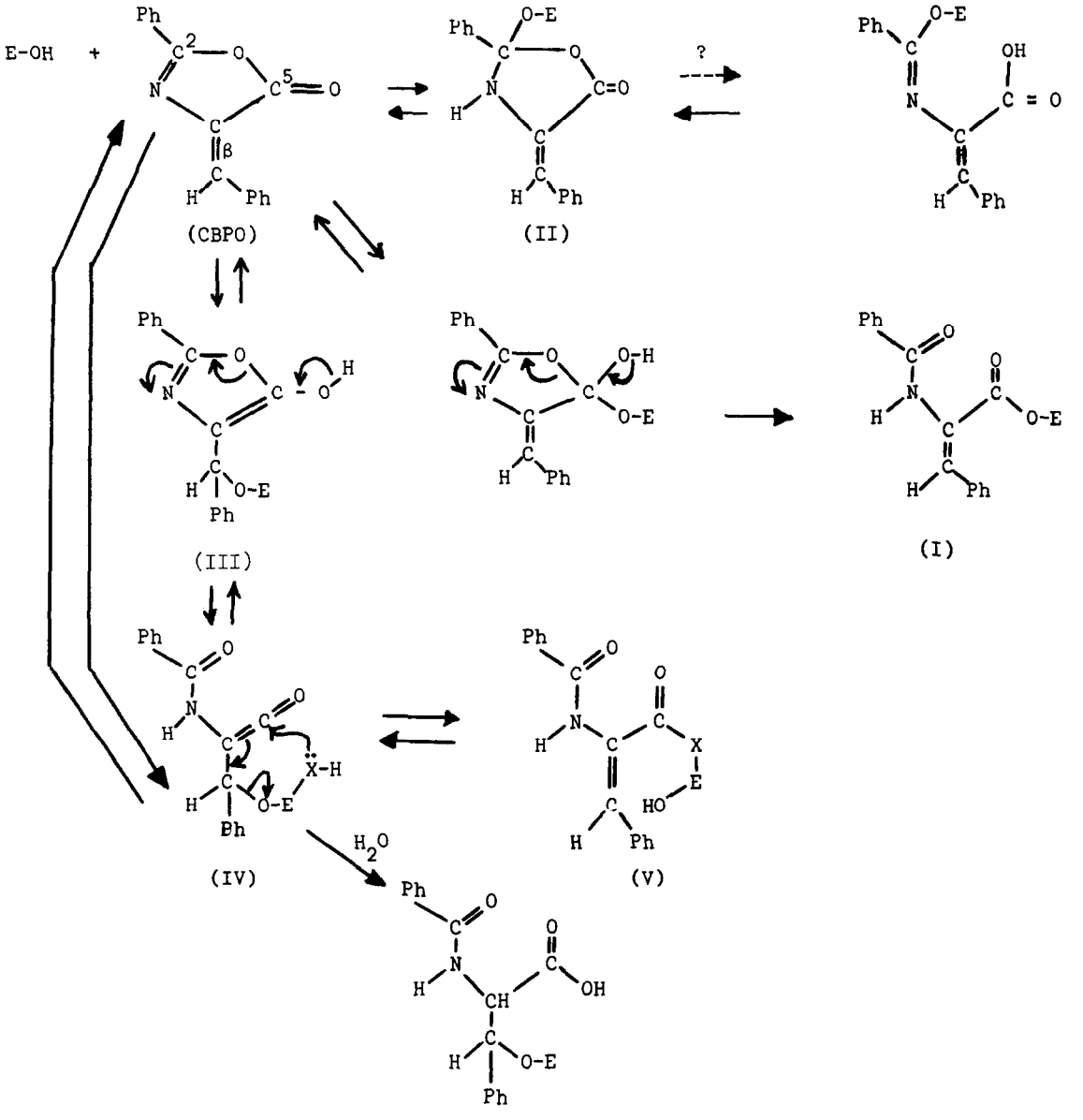
When the catalysis is carried out under non-Michaelis-Menten conditions with $[E] > [S_o]$, ES′ and ES″ are formed rapidly and since k_3 is measured directly as the first order rate constant of the slow deacylation step, it will not be subject to error in the enzyme concentration produced by non-productive binding. This error in the enzyme concentration, however, will be reflected in k_{cat} which is measured not directly as a first order rate constant but by measuring a rate (V_{max}) and subsequently dividing this by the enzyme concentration.

Possible mechanistic interpretations of the kinetic scheme (2) are shown in Scheme (3). Since the acylation of the enzyme at least under conditions where $[E] > [S_o]$ appears to be in the vicinity of the active centre of the enzyme [2] the mechanisms have been written in terms of the reaction of one nucleophilic centre in the enzyme presumably the hydroxyl group of serine 195. It is possible, however, that the formation of the productive and non-productive enzyme-substrate compounds involves the reaction of different nucleophilic centres in the enzyme. The formation of α-benzamido-*cis*-cinnamoyl-α-chymotrypsin (I) presumed to be ES′ involves the attack of the nucleophilic centre of the enzyme at C_5 of the oxazolinone ring. It is there suggested that ES″, the covalent non-productive compound, is the tetrahedral addition compound (II) resulting from the attack of the nucleophilic centre in the enzyme at C_2 of the oxazolinone ring. Attack of nucleophiles at C_2 of oxazolinones is not unprecedented; e.g. this is the electrophilic centre in 4,4-dimethyl oxazolin-5-one which is attacked by the hindered nucleophile, methyl α-aminoisobutyrate [10]. The required spectrophotometric characteristic of ES″ that it should lack ultraviolet absorption at 360 nm could be provided also by attack of the nucleophilic centre of the enzyme at the β-carbon atom of the benzylidene moiety to give the non-absorbing substituted ketene (IV). This could form either directly or through the intermediacy of the addition of compound (III) which would probably absorb to some extent at 360 nm.

Compound (IV), however, would probably undergo rapid spontaneous hydrolysis unless a nucleophilic centre (X) in the enzyme were aligned for reaction with the electrophilic centre of the ketene in which case an α-benzamido-*cis*-cinnamoyl enzyme (V) could result.

We have shown previously [8] that for the kinetic scheme (2) if it is assumed that $k_1 \gg k_2$, k_{cat} and k_3 are related by eq. (1) in which the other parameters are defined by eqs. (2)–(4).

$$k_3 - k_{cat} = \frac{k_3 K_m(\text{app}) \{K_s + K'_s\}}{K_s K'_s} \qquad (1)$$



Scheme (3).

$$K_m(\text{app}) = k_3 K_s / \{ k_3 K_s / K'_s) + k_2 + k_3 \} \quad (2)$$

$$K_s = k_1 / k_1 \quad (3)$$

$$K'_s = k'_1 / k'_1 \, . \quad (4)$$

Using for the catalysis at pH ca. 7.9 the measured values of $k_3 \cong 0.13$ sec^{-1} and $k_{cat} \cong 0.03$ sec^{-1} the expression on the RHS of eq. (1) has the value 0.1 sec^{-1}. Using the measured value of K_m(app) =

Table 2
Computed values of K_s and K'_s.

k_2, sec^{-1}	K_s, M	K'_s, M
0.005	5.01×10^{-7}	2.5×10^{-4}
0.05	5.1×10^{-7}	2.55×10^{-5}
0.1	10^{-6}	10^{-6}
1	10^{-5}	5×10^{-7}
10	10^{-4}	5×10^{-7}
10^2	10^{-3}	5×10^{-7}
10^3	10^{-2}	5×10^{-7}
10^4	10^{-1}	5×10^{-7}
10^5	1	5×10^{-7}

Values of k_2 are arbitrary and K_s and K'_s are computed from eqs. (5) and (7).

4×10^{-7}, $k_3 K_m(\text{app}) = 5.2 \times 10^{-8}$ sec^{-1}. Eq. (5) results from substitution of these measured values of k_{cat}, k_3 and K_m(app) into eq. (1).

$$K_s K'_s/(K_s + K'_s) \cong 5 \times 10^{-7} \text{ M} \quad (5)$$

Eq. (6) can be shown to hold for Scheme (2) (see [8]) and substitution of the measured values of k_{cat}, and K_m(app) into eq. (6) gives eq. (7).

$$k_{cat}/K_m(\text{app}) = k_2/K_s \quad (6)$$

$$K_2/K_s \cong 8 \times 10^4 \cong 10^5 \text{ } M^{-1} \text{ } sec^{-1} \quad (7)$$

By choosing a range of values for k_2, the corresponding values of K_s and K'_s, the respective productive and non-productive binding constants, may be computed from eqs. (5) and (7). These values are presented in table 2. Since it seems likely that $k_2 >$ ca. 1 sec^{-1}, the probable value of K'_s is ca. 5×10^{-7} M which is closely similar to the measured value of K_m(app).

Experimental

CBPO was prepared by literature methods e.g. that of Gillespie and Snyder [11] and after recrystallisation from benzene had m.p. 167–167.5° (ref. [11] gives m.p. 167°). α-Chymotrypsin was the 3× crystallized product of Seravac Laboratories (Pty) Ltd., Moneyrow Green, Holyport, Maidenhead, Berks., England, and was used without further purification. The enzyme concentrations referred to are the concentrations of active enzyme determined by titration with N-*trans*-cinnamoylimidazole [12]. Acetonitrile was the spectro grade product of the Eastman Kodak Co. Ltd.

Kinetic measurements were made spectrophotometrically at 25.0° using a Cary 15 recording spectrophotometer. First order rate constants were computed from conventional infinity plots.

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