

The Actinidin-Catalysed Hydrolysis of *N*- α -Benzyloxycarbonyl-L-lysine *p*-Nitrophenyl Ester pH Dependence and Mechanism

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1. The hydrolysis of *N*- α -benzyloxycarbonyl-lysine *p*-nitrophenyl ester catalysed by the thiol protease, actinidin, from *Actinidia chinensis* has been studied under steady state and non-steady state ($[E]_0 > [S]_0$) conditions.

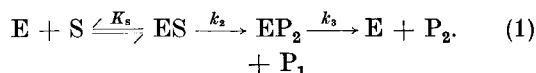
2. The Michaelis constant K_m is dependent on ionising groups of pK_a 3.75 and 8.1 and lowest at neutral pH. The catalytic centre activity, k_{cat} , is almost pH independent below pH 7.0 but increases greatly at high pH with a pK_a of 8.1.

3. A biphasic reaction was observed under conditions of $[E]_0 > [S]_0$ indicating that the simple acylation-deacylation mechanism does not apply and at least one additional step is necessary. The rapid phase of reaction involves production of *p*-nitrophenol bound to an acylated enzyme; *p*-nitrophenol is released in the slow phase.

4. From the amplitude and rate constant of the rapid phase, and the steady state parameters, the rate and equilibrium constants of some of the steps of the reaction were determined.

5. The biphasic behaviour under conditions of excess enzyme was found from pH 5.0 to 7.9 but the reaction was monophasic at pH 4.0.

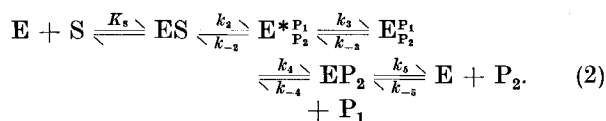
The mechanism of ester hydrolyses catalysed by the thiol proteases, papain and ficin, consists of at least three steps [1], involving a thioacyl enzyme intermediate (EP_2):



Steady state kinetic evidence [2] indicated that the papain-catalysed hydrolysis of the methyl, benzyl and *p*-nitrophenyl esters of benzyloxycarbonyl-lysine conformed to the above mechanism, where the alcohol (P_1) is released in the acylation (k_2) step and benzyloxycarbonyl-lysine (P_2) in the deacylation (k_3) step.

However rapid kinetic studies of the papain- and ficin-catalysed hydrolyses of the *p*-nitrophenyl ester (Z-Lys-ONp) [3] have shown that mechanism (1) is too simple to explain the non-steady state behaviour. The mechanism proposed involves a rate-limiting conformation change in the acyl-enzyme

before release of the leaving group from the active site:



The rate-limiting step is the k_3 step, while k_4 represents the rapid release of P_1 from the enzyme and k_5 represents the deacylation step.

The thiol protease, actinidin [4], from the berries of *Actinidia chinensis* (chinese gooseberries) has been shown to have many similarities to ficin and papain in steady state kinetic behaviour, specificity and physical properties [5–7]. We have studied the actinidin-catalysed hydrolysis of Z-Lys-ONp in order to determine further similarities in pH dependence and detailed mechanism.

The present communication reports the pH dependence and non-steady state kinetics of the actinidin-catalysed hydrolysis of Z-Lys-ONp. A biphasic reaction was observed under conditions of excess enzyme indicating that scheme (1) is too

Abbreviations. Z-Lys-ONp, *N*- α -benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester.

Enzymes. Ficin (EC 3.4.4.12); papain (EC 3.4.4.10).

simple. The data are, however, consistent with scheme (2) and the values of some of the rate constants involved are given.

EXPERIMENTAL PROCEDURE

Materials

Z-Lys-ONp (Cyclo), dithioerythritol (Sigma) and sodium tetrathionate (Fluka) were used without further purification. Actinidin was prepared as described previously [5], without rechromatography. The enzyme concentration was determined from assays with 0.1 mM Z-Lys-ONp at pH 6.0, assuming that $k_{\text{cat}} = V/[E] = 29 \text{ s}^{-1}$ [5].

Methods

Stopped-flow experiments were carried out using a Durrum-Gibson D110 stopped-flow spectrophotometer (Durrum Instruments Corp., Palo Alto, California, U.S.A.) and a Hewlett-Packard model 141B storage oscilloscope. Slower reactions were followed on a Perkin-Elmer PE402 spectrophotometer using a Smiths Servoscribe recorder.

Steady-State Experiments, $[S]_0 \gg [E]_0$

Hydrolyses of Z-Lys-ONp at pH 7.5 and below were followed at 348 nm as described previously [5]. Reactions above pH 7.5 were followed at 348 nm on the stopped-flow spectrophotometer, using higher enzyme concentrations, since base-catalysed hydrolysis of the substrate becomes very rapid at high pH. Acetate buffers were used from pH 3.5 to 5.5, phosphate buffers from pH 5.8 to 7.5 and Tris-HCl buffers from pH 7.8 to 8.8. All buffers were of ionic strength 0.1 and were prepared according to standard procedures [8]. Experiments at each pH were carried out in triplicate and the kinetic parameters, V and K_m , determined for each run. Velocities were obtained by the chords method [9], corrected for non-enzymic hydrolysis, and fitted to an Eadie plot [10] of v against $v/[S]$ by an unweighted least-squares program on the IBM 1130 computer. The catalytic centre activity, k_{cat} , was obtained from $V/[E]$.

Non-Steady-State Experiments, $[E]_0 > [S]_0$

Reactions were carried out in the stopped-flow instrument. One syringe contained a concentrated solution of reactivated [5] enzyme (50 to 800 μM), previously dialysed against an excess of buffer of the desired pH; the other contained an aqueous solution of Z-Lys-ONp, nominally 10 μM .

All quantitative experiments were at pH 7.0. Reactions were followed in the absorbance mode at 400 nm, typically for the first 50 ms. After reaction was complete, the oscilloscope was again triggered

to display the final absorbance. Oscilloscope traces were photographed on 35-mm film and subsequently projected onto graph paper from which data were obtained.

A gradual increase in the initial absorbance of the reaction mixture was observed during a series of runs (*i.e.* one 2 cm³ drive syringe full of each reactant). This increase, up to an absorbance of 0.01, was accompanied by a corresponding decrease in the amplitude of the observed reaction. Because of the high enzyme concentrations and low substrate concentration used, a small amount of reaction mixture entering the substrate syringe could contain sufficient enzyme to produce a slow breakdown of substrate to coloured product. The observed increase in absorbance would require less than 0.01 mm³ of reaction mixture to enter the substrate syringe during a series of reactions. The reaction mixture may be carried back either by diffusion or as a result of a reflected shock wave from the stoppage of flow.

In order to correct for this gradual decrease in substrate concentration with time, $[S]_0$ was determined before and after each experiment by extrapolation to zero time of a reaction at very low enzyme concentration ($\approx 0.5 \mu\text{M}$). Since the time delay between experiments was always approximately the same, the true $[S]_0$ is assumed to be the average of the $[S]_0$ values determined before and after each experiment.

Qualitative experiments at lower pH were followed at 320 nm and those at pH 7.9 followed at 400 nm. Oscilloscope traces were recorded as described above.

RESULTS AND DISCUSSION

Steady-State Experiments: pH Dependence of k_{cat} and K_m

The pH dependence of K_m is shown in Fig. 1; the data fit a normal curve with two ionising groups of $\text{p}K_a$ 3.75 ± 0.1 and 8.1 ± 0.1 . These values are comparable to the values of 4.30 and 8.0 found for k_{cat}/K_m of the papain-catalysed hydrolysis of Z-Lys-ONp [2]. Since both enzymes are known to have reactive cysteine residues, it is likely that actinidin has a cysteine-histidine pair responsible for catalytic activity as suggested for papain [11].

The pH profile for k_{cat} (Fig. 2) is unusual. The slight downward trend at low pH is not marked enough to represent the ionisation of a single group. At high pH k_{cat} increases with an apparent $\text{p}K_a$ of 8.1 ± 0.1 ; the ionising group involved may be that controlling K_m , which is likely to be the catalytic cysteine residue [11], although how it could cause this effect is unknown. Such behaviour has not been reported for other thiol proteases.

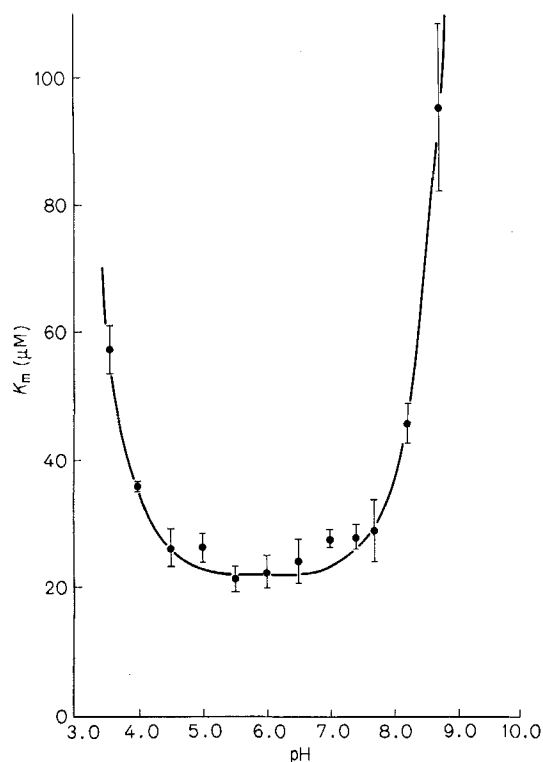


Fig.1. pH Dependence of K_m for the actinidin-catalysed hydrolysis of Z-Lys-ONp. $[S]_0 = 100 \mu\text{M}$, $I = 0.1$, 25°C . The solid line is the theoretical curve for two ionising groups of pK_a values 3.75 and 8.1 and $K_m(\text{min}) = 22 \mu\text{M}$

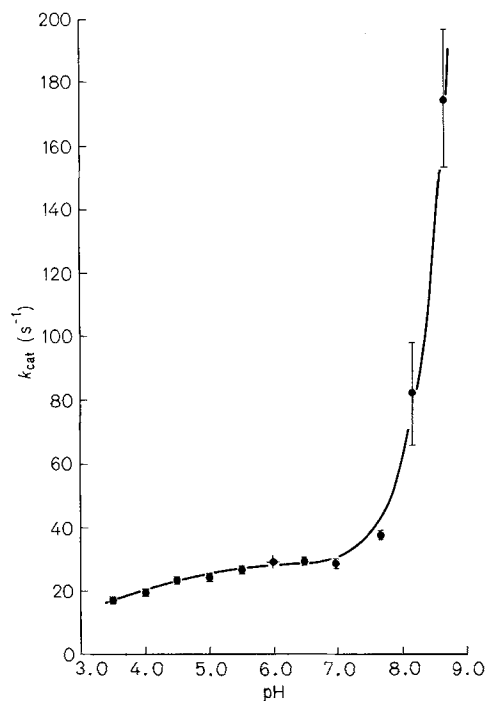
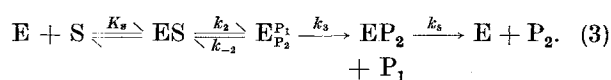


Fig.2. pH Dependence of k_{cat} for the actinidin-catalysed hydrolysis of Z-Lys-ONp. $[S]_0 = 100 \mu\text{M}$, $I = 0.1$, 25°C

Non-Steady-State Experiments

A typical experiment with $[E]_0 > [S]_0$ is shown in Fig.3. The reaction is first observed at the lowest point on the trace and a rapid increase in absorbance occurs to about 80% of the total absorbance change, followed by a slow increase to 100%. The biphasic nature of this reaction cannot be explained by the simple mechanism (1) since this predicts that the production of *p*-nitrophenolate ion (as P_1) should be a single-phase, first-order process [12].

These results can be fully explained by the more complex mechanism (2) but it is necessary to simplify the mechanism for the purpose of obtaining rate constants:



In this scheme, which is sufficient to explain the observed behaviour, the k_4 step of scheme (2) is omitted since it will probably be diffusion-controlled and sufficiently fast to have no effect on the observed rate constants. The reverse steps represented by k_{-4} and k_{-5} will be insignificant because they are

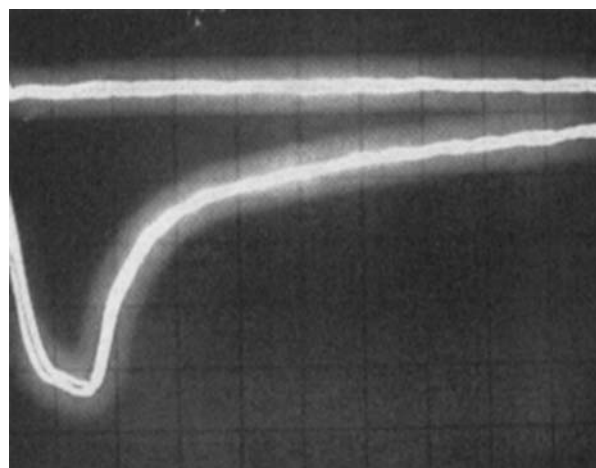


Fig.3. Time course of the absorbance change at 400 nm during the actinidin-catalysed hydrolysis of Z-Lys-ONp under conditions of $[E]_0 > [S]_0$. Substrate was nominally $5 \mu\text{M}$ and enzyme was $170 \mu\text{M}$; buffer was phosphate pH 7.0, $I = 0.1$. Vertical scale: 0.01 absorbance units per div, horizontal scale: 5 ms per div, time constant: 0.1 ms. The lowest grid line represents an absorbance of about 0.4. Three reactions are superimposed

second-order reactions and the concentrations of reactants will be very low. This argument is supported by the absence of product inhibition for this reaction.

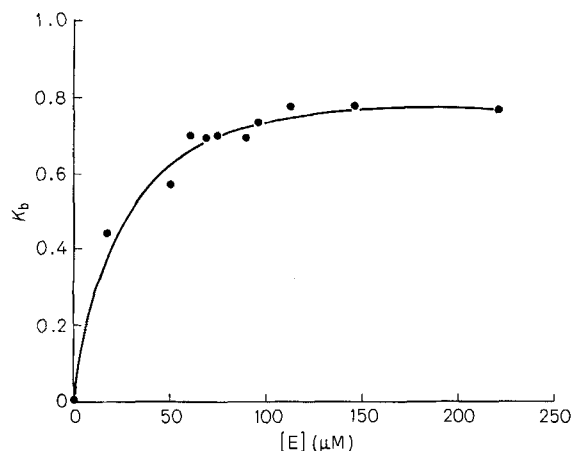


Fig. 4. The variation of K_b with enzyme concentration. $[S]_0 \approx 5 \mu\text{M}$, pH 7.0 ($I = 0.1$), 25°C

The k_3 step is regarded as effectively irreversible with the product reacting *via* the k_4 step as soon as it is produced since the k_{-3} step is not likely to be nearly as rapid as a diffusion-controlled reaction, the k_4 step. Furthermore there is no evidence for a k_{-3} step in the actinidin-catalysed hydrolysis of Z-Lys-ONp. Alternatively this scheme may represent a mechanism where *p*-nitrophenol release (as the k_3 step) is rate determining, followed by a rapid deacylation step¹.

Using mechanism (3) the reaction can be interpreted as follows. The rapid part of the biphasic reaction consists of substrate binding (which should be very rapid) and the setting up of an equilibrium about the k_2 step. The subsequent slow phase of the reaction shows the removal of $E_{P_1}^{P_1}$ by the slow k_3 step, with concurrent production of P_1 .

At each enzyme concentration, the absorbance change in the rapid phase of reaction, the "burst", was determined by extrapolation to zero time of the slow phase, which was slow enough to deviate very little from linearity. The size of the burst, expressed as a fraction, K_b , of the total observed absorbance change, is plotted against enzyme concentration in Fig. 4. K_b increases with increasing enzyme concentration to a maximum of about 0.8. This behaviour is comparable to that of the ficin- and papain-catalysed hydrolyses of Z-Lys-ONp [3] where the magnitude of the slow phase decreased to a minimum with increasing enzyme concentration.

The observed absorbance is the sum of those of the two species $E_{P_1}^{P_1}$ and P_1 ; while the absorption coefficient of P_1 is known from independent experiments, that of $E_{P_1}^{P_1}$ is not. The failure of the "burst" to reach 100% of the total absorbance at saturating enzyme concentration may be due to a difference in

absorption coefficients. Under this explanation, all of the substrate is converted in the rapid phase to $E_{P_1}^{P_1}$, which has a lower absorption coefficient than P_1 ; the slow phase is due to the increase in absorbance on conversion to P_1 . An alternative explanation is that the absorption coefficients are similar but not all of the substrate is converted to $E_{P_1}^{P_1}$ in the rapid phase, even at very high $[E]_0$.

We consider the second explanation more reasonable, since the evidence suggests that $E_{P_1}^{P_1}$ and P_1 have the same absorption coefficient. It is implied in mechanism (3) that $E_{P_1}^{P_1}$ consists of an thioacyl intermediate in which the *p*-nitrophenolate ion is bound to the enzyme in some unspecified way. This binding is not covalent since $E_{P_1}^{P_1}$ absorbs strongly at 400 nm, producing the rapid absorbance change; a covalent intermediate should have a low absorbance similar to that of a *p*-nitrophenyl ester, such as Z-Lys-ONp. Since the pK_a of *p*-nitrophenol is close to pH 7.0 a microenvironmental perturbation of pH in the active site could cause a change in absorption coefficient by changing the proportions of *p*-nitrophenol and the *p*-nitrophenolate ion. However when the hydrolysis of Z-Lys-ONp was followed at 320 nm, where *p*-nitrophenol absorbs strongly, the shape of the absorbance *versus* time curve was identical to that at 400 nm, indicating that a microenvironmental pH effect was absent. A final possibility is that the hydrophobic active site may reduce the absorption coefficient of the *p*-nitrophenolate ion. To test this various amounts of less polar solvents (ethanol, methanol and acetone) were added to an aqueous solution of *p*-nitrophenol at pH 7.0. The absorbance peak at 400 nm shifted in wavelength by less than 10% of the width at half height and there was only a slight increase in absorption coefficient. Therefore it is unlikely that the absorbance of P_1 bound to the enzyme in $E_{P_1}^{P_1}$ is greatly different from that in free solution.

If the absorption coefficients of $E_{P_1}^{P_1}$ and P_1 are equal, K_b is given by Eqn (4) (see Appendix for derivation):

$$K_b = \frac{1}{1 + \frac{k_{-2}}{k_2} + \frac{k_{-2}K_s}{k_2[E]_0}} \quad (4)$$

where $k_{-2} = k_{-2} + k_3$. The value of K_b at very high $[E]_0$ will be less than 1 if k_{-2} is a significant fraction of k_2 . Eqn (4) can be rearranged to give Eqn (5) which predicts that a plot of $1/K_b$ against $1/[E]_0$ will be a straight line.

$$\frac{1}{K_b} = \frac{1}{[E]_0} \cdot \frac{k_{-2}K_s}{k_2} + \frac{k_{-2}}{k_2} + 1 \quad (5)$$

$1/K_b$ is plotted against $1/[E]_0$ in Fig. 5; only results at high $[E]_0$ were used since these are the most useful for defining the required intercept. The best fit straight line (obtained by an unweighted least-

¹ We thank a referee for this suggestion.

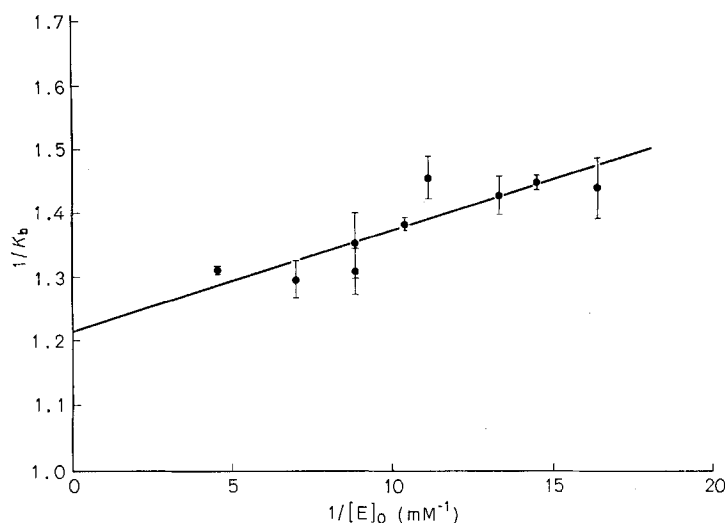


Fig.5. The variation of $1/K_b$ with $1/[E]_0$. $[S]_0 \cong 5 \mu\text{M}$, pH 7.0, $I = 0.1$, 25°C . Error bars represent the possible range of $1/K_b$ due to substrate breakdown between experiments (see Experimental Procedure). The solid line was obtained by the least-squares method

squares method) has an intercept on the y axis of 1.22 ± 0.04 , giving a k_α/k_2 value of 0.22 ± 0.04 , and a slope of $1.50 \pm 0.3 \times 10^{-5} \text{ M}$. The expression for the steady state parameter, k_{cat} , in terms of mechanism (3) is given in the Appendix.

$$k_{\text{cat}} = \frac{k_2 k_3}{k_2 + k_\alpha} \quad (6)$$

Using the value of k_{cat} in Table 1, and $k_\alpha/k_2 = 0.22$, values of k_3 and K_s were determined from Eqns (6) and (5) respectively and are given in Table 1.

The pseudo-first order rate constant for the rapid phase of reaction, k_φ , was obtained at various enzyme concentrations by extrapolating the slow phase to zero time and plotting the logarithm of the difference between the measured absorbance and extrapolated absorbance against time [13]. Eqn (7), derived in the Appendix, gives the relation between k_φ and the individual constants of scheme (3):

$$k_\varphi = \frac{k_2 [E]_0}{K_s + [E]_0} + k_\alpha \quad (7)$$

The values of k_2 were calculated for a series of six experiments at different enzyme concentrations and the average value is given in Table 1. The deviation given takes into account only the variation in k_φ values and the true error may be higher, up to 33%. k_{-2} was obtained from the values of k_2 and k_3 and the ratio k_α/k_2 . Since there is substantial error in k_2 and k_α/k_2 , the error in the value of k_{-2} , given in Table 1, is very large. Consequently the values of k_2 and k_{-2} given should be regarded mainly as an indication of their magnitudes.

Table 1. Kinetic parameters for the actinidin-catalysed hydrolysis of *Z-Lys-ONp* at pH 7.0 and 25°C

Individual rate and equilibrium constants are defined in terms of mechanism (3). All parameters were determined as described in the text

Parameter	Value	Unit
K_m	25 ± 2	μM
k_{cat}	29 ± 1	s^{-1}
K_s	83 ± 40	μM
k_2	400 ± 70	s^{-1}
k_{-2}	53 ± 40	s^{-1}
k_3	36 ± 1	s^{-1}

Three principal factors cause the experimental results to be rather imprecise.

a) The low substrate concentration used produces a total absorbance change of less than 0.1.

b) Background absorbance due to the enzyme is considerable at high $[E]_0$.

c) At the highest enzyme concentrations the rapid phase of reaction is almost complete within the 2 ms dead time of the apparatus, while at the lower concentrations the two phases of reaction are not clearly separated. In the latter case a significant amount of the E_P^1 produced is removed by the k_3 step and the integral term, which was ignored in the derivation becomes important.

Since the derivation of Eqns (4) and (7), used to obtain the rate constants in Table 1, involved approximations, the results were tested by simulation of the experimental progress curves. Digital analog simulation of scheme (3) was carried out on the IBM

1130 computer using an extended precision CSMP package and assuming that the absorption coefficients of E_2^P and P_1 were equal. The simulated curves showed a good fit to the experimental curves.

Effect of pH on Non-Steady-State Kinetics

Experiments with $[E]_0 > [S]_0$ were carried out at pH values from 4.0 to 7.9. Biphasic progress curves were observed throughout the range 5.0 to 7.9 but at pH 4.0 the reaction appeared monophasic, with rate constant about 20 s^{-1} . This suggests that k_2 and k_3 are of similar magnitude at pH 4.0, with an ionisation in k_2 of pK_a about 5. The low absorption coefficient of *p*-nitrophenol at 320 nm made the determination of k_2 at acidic pH impossible. However the acylation of ficin by *p*-nitrophenyl hippurate depends on an ionising group of pK_a 5.78 [14], suggesting that an ionisation in this range is reasonable for the corresponding step of the actinidin-catalysed hydrolysis of Z-Lys-ONp.

Rate of the Deacylation Step

The deacylation step, k_5 , has not been considered so far because it has no bearing on the non-steady state kinetics. It is, however, implicit in the derivation of Eqn (6) that k_5 is rapid and makes no contribution to k_{cat} . This was tested by determining k_{cat} in the presence and absence of 0.1 M L-tryptophanamide at pH 6.0 and 7.4. The value of k_{cat} remained unaltered, within experimental error, although L-tryptophanamide is known to produce significant enhancement of the rate of deacylation of an acyl-papain at concentrations below 0.1 M [15]. This indicates that k_5 is at least 3–4 times greater than k_3 , that is greater than 100 s^{-1} .

CONCLUSION

This communication reports the first direct observation of a biphasic process in an ester hydrolysis catalysed by a thiol protease under conditions of excess enzyme. In the previous study of the ficin- and papain-catalysed hydrolyses of Z-Lys-ONp [3], the rapid phase was too fast to be observed and the biphasic nature of the reaction was inferred from the variation with enzyme concentration of the amplitude of the observed part of the reaction. The value of k_2 for the actinidin-catalysed hydrolysis is still very high (400 s^{-1}) and the "burst" is only brought into the observable time range by a high value of K_s and the consequent lack of saturation at the enzyme concentrations used (Eqn 7).

The results presented here show conclusively that the simple acylation-deacylation mechanism (1) must be expanded by at least one step for the actinidin-catalysed hydrolysis of Z-Lys-ONp. The mechanism proposed for the papain- and ficin-catalysed hydrolyses [3] is consistent with the data for the

actinidin-catalysed hydrolysis and this is reasonable in view of the many similarities between these enzymes [5–7]. Structural studies being carried out in this laboratory [16] may help to explain the close correspondence between actinidin and papain.

Some final comments may now be made about some of the individual steps of the actinidin-catalysed hydrolysis of Z-Lys-ONp.

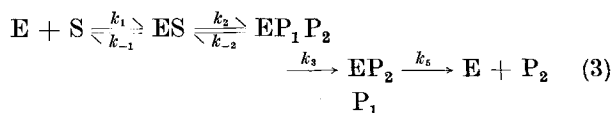
a) The acylation rate constant (k_2) of about 400 s^{-1} is not surprisingly large since excess free cysteine will attack the substrate with an acylation rate constant of $0.58 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.0 (M. J. Boland, unpublished results). The observed k_2 therefore corresponds to an effective SH concentration of 660 M in the active site, which can be readily accounted for by either orbital steering [17] or loss of entropy [18]. The relative sizes of k_2 and k_{-2} presumably reflect the free energy change for the acylation step which favours E_2^P by about 5 kJ/mol.

b) It is difficult to ascribe a function to the k_3 step. Since it is neither the acylation nor the deacylation step, it may represent an isomerisation of the acyl-enzyme. The involvement of an isomerisation as the rate-determining step has been demonstrated for the oxidation of lactate by lactate dehydrogenase [19]. Alternatively this step may represent slow diffusion of *p*-nitrophenol or *p*-nitrophenolate ion from the acyl-enzyme. In this case the increase in k_{cat} observed at high pH can be explained by repulsion of the *p*-nitrophenolate ion by the negatively charged cysteine side chain above the pK_a of the latter. However there is no obvious reason why *p*-nitrophenol release should be as slow as 36 s^{-1} .

c) The k_5 step, the hydrolysis of the thioacyl enzyme, is the step about which least is known since it occurs after the rate-determining step. However it is probably the step where enzymic catalysis is most important as the deacylation step is usually the slowest step in model systems. A histidine residue is likely to act as a general base catalyst in the deacylation step and an isomerisation in the acyl-enzyme may activate the thioester bond by distortion as in Lumry's rack mechanism [20]. Although the k_3 step is rate limiting for the actinidin-catalysed hydrolysis of Z-Lys-ONp, the deacylation step is likely to be the slow step for less labile substrates. Therefore consideration of the sources of catalysis by actinidin and other thiol proteases should centre on the deacylation reaction and the role of isomerisations of the acyl-enzyme in assisting this reaction.

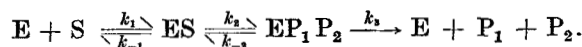
APPENDIX

Derivation of Kinetic Parameters for Mechanism (3)



Steady-State Conditions ($[S]_0 \gg [E]_0$)

If $k_3 \ll k_5$, i.e. deacylation is rapid, then this scheme can be reduced to:



For this scheme the following expression for k_{cat} can be derived (cf. [21]):

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_{-2} + k_3}. \quad (8)$$

Replacing $k_{-2} + k_3$ by k_α , this becomes

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_\alpha}. \quad (6)$$

Non-Steady-State Conditions ($[E]_0 [S]_0$)

Provided that binding is rapid

$$[S] = \frac{[ES] K_s}{[E]}$$

where K_s is the enzyme-substrate dissociation constant [22]. At any time after reaction commences the sum of the concentrations of various species is equal to the initial substrate concentration.

$$[S]_0 = [S] + [ES] + [EP_1P_2] + [P_1]$$

$$= [ES] \left(1 + \frac{K_s}{[E]}\right) + [EP_1P_2] + [P_1]$$

$$\therefore [ES] = \frac{[S]_0 - [EP_1P_2] - [P_1]}{1 + K_s/[E]}.$$

The net rate of formation of EP_1P_2 is given by:

$$\begin{aligned} \frac{d[EP_1P_2]}{dt} &= k_2 [ES] - (k_{-2} + k_3) [EP_1P_2] \\ &= \frac{k_2 ([S]_0 - [EP_1P_2] - [P_1])}{1 + K_s/[E]} - k_\alpha [EP_1P_2] \end{aligned}$$

where $k_\alpha = k_{-2} + k_3$

$$\begin{aligned} \therefore \frac{d[EP_1P_2]}{dt} &= \frac{k_2 [S]_0 [E]}{K_s + [E]} \\ &- \left(\frac{k_2 [E]}{K_s + [E]} + k_\alpha \right) [EP_1P_2] - \frac{k_2 [E] [P_1]}{K_s + [E]}. \quad (9) \end{aligned}$$

Since P_1 is formed from EP_1P_2 and not removed, the concentration at time t is given by:

$$[P_1] = \int_0^t k_3 [EP_1P_2] dt$$

$$\begin{aligned} \therefore \frac{d[EP_1P_2]}{dt} &= \frac{k_2 [S]_0 [E]}{K_s + [E]} \\ &- \left(\frac{k_2 [E]}{K_s + [E]} + k_\alpha \right) [EP_1P_2] \\ &- \frac{k_2 [E]}{K_s + [E]} \int_0^t k_3 [EP_1P_2] dt. \quad (10) \end{aligned}$$

If k_3 is small compared to k_2 (i.e. k_3 is rate-determining) and t is small (during the rapid phase),

there will be little production of P_1 and the integral term in Equation (10) will become insignificant.

Since $[E]_0 \gg [S]_0$, $[E]$ can be regarded as constant and equal to $[E]_0$

$$\begin{aligned} \therefore \frac{d[EP_1P_2]}{dt} &= \frac{k_2 [S]_0 [E]_0}{K_s + [E]_0} \\ &- \left(\frac{k_2 [E]_0}{K_s + [E]_0} + k_\alpha \right) [EP_1P_2]. \quad (11) \end{aligned}$$

This is of the form:

$$\frac{d[EP_1P_2]}{dt} = a - b [EP_1P_2].$$

Since $[EP_1P_2] = 0$ when $t = 0$, integration gives:

$$[EP_1P_2] = \frac{a}{b} (1 - e^{-bt}).$$

When t is large and the rapid phase almost complete, the exponential term will tend to zero. Therefore, $[EP_1P_2]$ will tend to a/b and the absorbance change in the burst, B , will be given by:

$$\begin{aligned} B &= \frac{a}{b} \varepsilon_{EP_1P_2} \\ &= \frac{k_2 [S]_0 [E]_0}{K_s + [E]_0} \varepsilon_{EP_1P_2} / \left(\frac{k_2 [E]_0}{K_s + [E]_0} + k_\alpha \right) \\ &= \frac{[S]_0 \varepsilon_{EP_1P_2}}{1 + \frac{k_\alpha}{k_2} + \frac{k_\alpha K_s}{k_2 [E]_0}}. \end{aligned}$$

The total absorbance change is equal to $[S]_0 \varepsilon_{P_1}$

$$\therefore K_b = \frac{B}{[S]_0 \varepsilon_{P_1}} = \frac{\varepsilon_{EP_1P_2}}{\varepsilon_{P_1} \left(1 + \frac{k_\alpha}{k_2} + \frac{k_\alpha K_s}{k_2 [E]_0} \right)}.$$

If $\varepsilon_{EP_1P_2} = \varepsilon_{P_1}$, this reduces to:

$$K_b = \frac{1}{1 + \frac{k_\alpha}{k_2} + \frac{k_\alpha K_s}{k_2 [E]_0}}. \quad (4)$$

The first-order rate constant for the rapid phase, k_φ , will be equal to b .

$$\text{i.e. } k_\varphi = \frac{k_2 [E]_0}{K_s + [E]_0} + k_\alpha. \quad (7)$$

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