Isomerization of the uncomplexed actinidin molecule: kinetic accessibility of additional steps in enzyme catalysis provided by solvent perturbation

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The effects of increasing the content of the aprotic dipolar organic co-solvent acetonitrile on the observed first-order rate constant (k_{obs}) of the pre-steady state acylation phases of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionester catalysed by the cysteine proteinase variants actinidin and papain in sodium acetate buffer, pH 5.3, were investigated by stopped-flow spectral analysis. With low acetonitrile content, plots of k_{obs} against $[S]_0$ for the actinidin reaction are linear with an ordinate intercept of magnitude consistent with a five-step mechanism involving a post-acylation conformational change. Increasing the acetonitrile content results in marked deviations of the plots from linearity with a rate minimum around $[S]_0 = 150 \mu\text{M}$. The unusual negative dependence of k_{obs} on $[S]_0$ in the range 25–150 μM is characteristic of a rate-determining isomerization of the free enzyme before substrate binding, additional to the five-step

mechanism. There was no evidence for this phenomenon nor for the post-acylation conformational change in the analogous reaction with papain. For this enzyme, however, acetonitrile acts as an inhibitor with approximately uncompetitive characteristics. Possible mechanistic consequences of the differential solvent-perturbed kinetics are indicated. The free enzyme isomerization of actinidin may provide an explanation for the marked difference in sensitivity between this enzyme and papain of binding site–catalytic site signalling in reactions of substrate-derived 2-pyridyl disulphide reactivity probes.

Key words: actinidin, cysteine proteinase mechanism, free enzyme isomerization, papain, solvent dependence of rate-determining step, uncompetitive inhibition.

INTRODUCTION

An essential part of the investigation of enzyme mechanism is kinetic analysis of rate-determining processes, while recognizing that other steps of the mechanism may exist that are normally inaccessible to kinetic study. Kinetic access can be widened if it can be arranged for different steps to be rate-determining in different circumstances. Obvious targets for study include variation in substrate structure, enzyme structure and reaction conditions. Well-known examples involving variation in structure include studies of the acylation and deacylation steps of serine-proteinase-catalysed hydrolysis by using amide and ester substrates respectively (see [1] for a review). This approach may be refined to include investigation of tetrahedral species within the acylation and deacylation processes of both serine proteinase and cysteine proteinase mechanisms by using Hammett analysis (see [1,2]). We have used the natural variation in the structure of members of the papain family of cysteine proteinases to reveal gradations in functional characteristics and mechanistic phenomena that are more easily demonstrated in some variants than in others [2–7]. In our most recent studies [6,7], we used the chromogenic substrate *N*-acetyl-Phe-Gly methyl thionester (methyl thionester **1**; Figure 1) to investigate the acylation and deacylation stages of the catalysis of its hydrolysis by the cysteine proteinase variants, papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14). Papain is the much-studied minor cysteine proteinase component of the latex of *Carica papaya* and actinidin is the cysteine proteinase from *Actinidia chinensis* (kiwi fruit or Chinese gooseberry). Evidence is accumulating that these enzymes lie at the extremes

of the spectrum of active-centre behaviour exhibited by several members of the papain family [2]. The specific thionoester substrate **1** contains the principal recognition sites for papain, i.e. the phenylalanine residue at P₂ to provide proper occupancy of the S₂ subsite and the P₁–P₂ amide (peptide) bond. It allows the pre-steady state formation of the dithioester intermediates (**2a** and **2b**; Figure 1) to be monitored at 315 nm on the stopped-flow time scale, consequent on the generation of the $-(C=S)-S$ -Enzyme chromophore. The subsequent disappearance of the A₃₁₅ signal allows the steady state deacylation phase of the catalysis to be monitored. The kinetic studies with a low organic co-solvent concentration reported in [6,7] provide evidence of differences between papain and actinidin. Thus, whereas for papain, in accord with the traditional view, the rate-determining step is the base-catalysed reaction of the acyl enzyme intermediate with water, for actinidin, it is a post-acylation conformational change required to permit release of the methanol product and its replacement in the catalytic site by the key water molecule whose reaction ultimately provides deacylation. The results obtained for actinidin may be described by the five-step model, eqn (1):

In this model, ES is the adsorptive complex and (ES'.P₁)* represents the conformation of the acyl–enzyme–alcohol product complex from which the alcohol diffuses rapidly into bulk solvent

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1 **X = O, R = CH₃**

2a **X = S, R = Papain**

2b **X = S, R = Actinidin**

Figure 1 Thionoester substrate (**1**) and dithioacyl enzyme intermediates (**2a** and **2b**)

to leave (ES')*. This is followed by the binding of water and deacylation to regenerate the enzyme, with the formation of the carboxylate product, P₂. The isomerization of the initial acyl–enzyme–alcohol product complex (ES'.P₁) to the conformer (ES'.P₁)* is considered to be rate-determining.

In the present paper, we report the results of experiments involving changing the reaction conditions for the hydrolysis of the thionoester **1** catalysed by actinidin and by papain, by increasing the concentration of the dipolar aprotic co-solvent, acetonitrile. As is well known (e.g. see [8]), co-solvents can produce localized changes in enzyme structure that may modify its reactivity under circumstances where no detectable general denaturation is observable. Induction of new protein conformations associated with changed rate-constant ratios for steps in the catalytic mechanism can be envisaged either by direct interactions of co-solvent and protein sites or by changes in water structure in the solvation shells. In the present paper, we report that increasing the acetonitrile concentration reveals an additional feature of the mechanism of the actinidin-catalysed hydrolysis of **1**, namely a rate-determining conformational change of the free enzyme molecule before adsorptive complex formation, a feature that could not be demonstrated for the corresponding papain-catalysed hydrolysis. We report also that acetonitrile inhibits the papain reaction in a manner that approximates to the relatively rare phenomenon of uncompetitive inhibition. Possible consequences for the cysteine proteinase mechanism are indicated.

MATERIALS AND METHODS

Enzymes, thionoester substrate (**1**) and organic co-solvent

Methods of purification of actinidin [9] and papain [10], which include the production of fully active enzymes by covalent chromatography, reviewed in [11], have been described previously, as has their active-centre evaluation by spectroscopic titration at 343 nm ($\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 2,2'-dipyridyl disulphide as titrant [12].

N-Acetyl-Phe-Gly methyl thionoester (compound **1**) was synthesized as described in [6] and characterized as described in [6,7]. Acetonitrile, the organic co-solvent used in the kinetic experiments was HPLC grade supplied by VWR International (Poole, U.K.).

Kinetic studies on the reactions of actinidin and papain with the thionoester substrate (**1**)

All reactions were carried out at 25 °C in sodium acetate buffer, pH 5.3 and I 0.1, containing 1 mM EDTA and 4.3–16.7% (v/v) acetonitrile with $[S]_0 > 10[E]_T$. Solvents were degassed under decreased pressure. Kinetic studies were performed with an Applied Photophysics SF.17MV stopped-flow spectrophotometer, kinetics workstation and data-acquisition and analysis software. Formation and subsequent hydrolysis of the dithioacyl enzyme intermediates (**2**) were monitored at 315 nm. The increase in A_{315} (over 0.2 s for the papain reactions and over 2 s for those of actinidin) provides the observed first-order rate constant (k_{obs}) for the formation of the dithioacyl enzymes. The rate constants were evaluated by fitting the A_{315}/t data, collected by the Acorn Archimedes microcomputer (Cambridge, Cambridgeshire, U.K.), of the stopped-flow system to the equation for a single exponential process:

$$A = P_1 e^{P_2 t} + P_3,$$

where

$$P_1 = |A_{\infty} - A_0|, P_2 = k_{\text{obs}} \text{ or } k \text{ and } P_3 = A_{\infty}.$$

Subsequent processing of kinetic results was carried out by using Sigmaplot 5.0 (Jandel Scientific, Erkrath, Germany). Estimates of the parameters of eqn (8) (see the Discussion section) were obtained by using a four-parameter rate equation written for inclusion in the Regression Wizard equation editor.

RESULTS

Characteristics of the hydrolysis of the methyl thionoester substrate (**1**) in aqueous acetonitrile, pH 5.3, catalysed by actinidin and papain

The general form of the time courses for the hydrolysis of substrate **1** in 0.1 M sodium acetate buffer, pH 5.3, containing 4.3%, 8.3%, 12.5% or 16.7% (v/v) acetonitrile catalysed by the two enzyme variants is the same as that reported previously [6,7] at various pH values in aqueous buffer containing 4.2% (v/v) DMSO. Thus, as expected, rapid pre-steady state formation of the dithioacyl enzyme (ES'), detected by an increase in A_{315} , is followed by slower steady-state loss of absorbance at this wavelength resulting from its hydrolysis (deacylation), which regenerates the catalytic site Cys-S⁻/His-Im⁺H ion pair. The dependence on $[S]_0$ of the observed first-order rate constant, k_{obs} , for the pre-steady state phase for the various solvent mixtures is shown in Figure 2 for the actinidin reaction and in Figure 3 for the papain reaction. The characterizing parameters of the latter are compared in Table 1.

DISCUSSION

General characteristics

The general form of the time courses is in accord with both the five-step model of eqn (1) for actinidin and the minimal model of eqn (2) for papain:

both discussed previously [6,7].

Ready analysis of these models requires the formation of the first transient (formation of adsorptive complex, ES) to be complete within the dead time of the stopped-flow system and the second transient, the formation of (ES'), to be slow enough to be

Figure 2 Dependence on substrate concentration of the observed first-order rate constant (k_{obs}) for the pre-steady state phase of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionester (1) catalysed by actinidin in aqueous acetonitrile

Reactions were carried out at 25 °C with $[S]_0 > 10[E]_T$ in sodium acetate buffer, pH 5.3, / 0.1, containing 1 mM EDTA and the following amounts (v/v) of acetonitrile: 4.3% (a), 8.3% (b), 12.5% (c) and 16.7% (d). The experimental points are mean values of five determinations. The S.D. values were at most $\pm 10\%$ of the means. The lower limit of $[S]_0 = 30 \mu\text{M}$ was dictated by the necessity (i) to use $[E]_T \geq 3 \mu\text{M}$ to provide good-quality progress curves and (ii) to satisfy the pseudo-first-order condition. In (a)–(c), the lines are theoretical for eqn (4) and the following parameter values: (a) $k_{+2}/K_{\text{m(acyl)}} = (1.7 \pm 0.03) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-2} + k_{+3} = 1.95 \pm 0.09 \text{ s}^{-1}$ from which $k_{-2} = 1.85 \text{ s}^{-1}$; (b) $k_{+2}/K_{\text{m(acyl)}} = (8.10 \pm 0.13) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-2} + k_{+3} = 2.62 \pm 0.04 \text{ s}^{-1}$ from which $k_{-2} = 2.52 \text{ s}^{-1}$; (c) $k_{+2}/K_{\text{m(acyl)}} = (7.90 \pm 0.03) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-2} + k_{+3} = 2.17 \pm 0.01 \text{ s}^{-1}$ from which $k_{-2} = 2.07 \text{ s}^{-1}$; in (d), the line is theoretical for eqn (8) and the following parameter values: $k_{+1} = 6.06 \pm 1.02 \text{ s}^{-1}$, $k_{-1} = 3.6 \times 10^{-9} \pm 1.2 \text{ s}^{-1}$ (this parameter is not well-defined by the data and is probably < 1), $k'_F = (7.2 \pm 1.6) \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$, $K_{\text{ES}'} = 116 \pm 45 \mu\text{M}$.

observed on the stopped-flow timescale. Exponential increase of $[ES']$, detected by an increase in A_{315} to its steady-state value, is followed by the rate-determining deacylation phase over a longer timescale. The latter eventually involves exponential decrease in A_{315} , which is independent of $[S]_0$ and is characterized by a first-order rate constant k , which is k_{+3} in both eqns (1) and (2). The present paper is concerned with the pre-steady state phase, the phase that responds markedly to variation in the composition of the solvent.

The pre-steady state (acylation) phase of the actinidin reaction

In terms of the model of eqn (1), when a reaction is carried out with $[S]_0 \gg [E]_T$, k_{obs} is predicted to vary with $[S]_0$ according to eqn (3) [6,7,13]:

$$k_{\text{obs}} = \frac{k_{+2}[S]_0}{K_{\text{m(acyl)}} + [S]_0} + k_{+3} + k_{-2} \quad (3)$$

where $K_{\text{m(acyl)}} = (k_{-1} + k_{+2})/k_{+1}$, which can approximate to $k_{-1}/k_{+1} = K_s$ [14]. Eqn (3) predicts that a plot of k_{obs} against $[S]_0$ will contain a hyperbolic component with ordinate intercept $k_{\text{obs}} = k_{+3} + k_{-2}$ when $[S]_0 = 0$. Eqn (3) differs from the cor-

Figure 3 Dependence on substrate concentration of the observed first-order rate constant (k_{obs}) for the pre-steady state phase of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionester (1) catalysed by papain in aqueous acetonitrile

The reaction conditions and the means and range of S.D. values were as in the legend to Figure 2 with the following contents (v/v) of acetonitrile: 4.3% (a), 8.3% (b) and 16.7% (c). In (a), the line is theoretical for eqn (4) with $k_{-2} = 0$ and $k_{+2}/K_{\text{m(acyl)}} = (5.4 \pm 0.2) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. In (b) and (c), the lines are theoretical for eqn (3) with $k_{-2} = 0$ and the following parameter values (b) $k_{+2} = 543 \pm 221 \text{ s}^{-1}$ and $K_{\text{m(acyl)}} = 1368 \pm 700 \mu\text{M}$; (c) $k_{-2} = 249 \pm 30 \text{ s}^{-1}$ and $K_{\text{m(acyl)}} = 893 \pm 148 \mu\text{M}$.

Table 1 Dependence of the kinetic parameters for the pre-steady-state acylation phase of the catalysis of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionester (1) catalysed by papain on solvent composition

For other reaction conditions, see the legend to Figure 3.

Co-solvent (v/v)	$k_{+2} (\text{s}^{-1})$	$K_{\text{m(acyl)}} (\mu\text{M})$	$k_{+2}/K_{\text{m(acyl)}} (\text{M}^{-1} \cdot \text{s}^{-1})$	Reference
4.2% DMSO	116 ± 14	184 ± 53	6.3×10^5	[6]
4.3% Acetonitrile	$\gg 250$	$\gg 500$	5.3×10^5	Present work
8.3% Acetonitrile	543 ± 221	1368 ± 700	4.0×10^5	Present work
16.7% Acetonitrile	249 ± 30	893 ± 148	2.8×10^5	Present work

responding equation for the model of eqn (2) only in having k_{-2} as an additional term. Thus when k_{obs} is plotted against $[S]_0$, there is an ordinate intercept with the value of $k_{+3} + k_{-2}$, whereas when irreversible formation of the acyl enzyme intermediate is assumed (eqn 2), the ordinate intercept has the value of k_{+3} . For the actinidin reaction, the value of k_{+3} is small ($0.1 \pm 0.01 \text{ s}^{-1}$) and so the ordinate intercept derives largely from the value of k_{-2} . Obviously, the ordinate intercept exists also for the condition $[S]_0 \ll K_{\text{m(acyl)}}$, when eqn (3) becomes eqn (4):

$$k_{\text{obs}} = \frac{k_{+2}}{K_{\text{m(acyl)}}} \cdot [S]_0 + k_{+3} + k_{-2} \quad (4)$$

Then, however, the hyperbolic component of the curve is replaced by an essentially linear response of slope $k_{+2}/K_{\text{m(acyl)}}$.

Figure 2 shows the dependence of the k_{obs} on $[S]_0$, for the pre-steady state phase of the reaction of the methyl thionester

substrate (**1**) with actinidin in 0.1 M sodium acetate buffer, pH 5.3, containing acetonitrile [4.3 % (v/v) (a), 8.3 % (v/v) (b), 12.5 % (v/v) (c) or 16.7 % (v/v) (d)]. The values of the characterizing parameters are given in the Figure legend. The plots for the reactions in 4.3 % and 8.3 % acetonitrile, Figures 2(a) and 2(b) respectively, are similar to each other and to the plot for the reaction in 4.2 % (v/v) DMSO reported in [6]. Thus the plots, which correspond to eqn (4), are linear with slopes $k_{+2}/K_{m(\text{acyl})}$ approx. $1-2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and ordinate intercepts minus k_{+3} (0.1 s^{-1}) (i.e. k_{-2}) approx. $2-3 \text{ s}^{-1}$. The particularly interesting features occur at higher acetonitrile concentrations, in Figures 2(c) and 2(d). In Figure 2(c) (12.5 % acetonitrile), the deviation from linearity suggests the possibility of either a plateau or a minimum in the region $[S]_0 < 200 \mu\text{M}$ and the existence of a minimum around $[S]_0 = 150 \mu\text{M}$ is demonstrated clearly in Figure 2(d) (16.7 % acetonitrile). The decrease in k_{obs} (and $k_{\text{obs}} - k_{+3}$) with increase in $[S]_0$ in the range $25-150 \mu\text{M}$ is a striking result. Such negative-dependence of k_{obs} on substrate concentration is comparatively rare (e.g. see [15,16]) and is characteristic of isomerization of the free enzyme ($F \rightleftharpoons E$) before substrate binding. The minimal model for this phenomenon involving a covalent intermediate and a free enzyme isomerization is eqn (5):

The observed first-order rate constant (k_{obs}) for the formation of the acyl enzyme intermediate in terms of the model of eqn (5) when (i) deacylation (k_{+3}) is slow and (ii) the isomerization step is slower than the substrate-processing step, with the E to ES' step rapidly equilibrating, is given by eqn (6) [15–17]:

$$k_{\text{obs}} = \frac{k_{-1}K_{ES'}}{K_{ES'} + [S]_0} + k_{+1} \quad (6)$$

where $K_{ES'} = [E][S]/[ES' \cdot P_1]$.

Eqn (6) shows that, as $[S]_0 \rightarrow \text{zero}$, $k_{\text{obs}} \rightarrow k_{-1} + k_{+1}$, i.e. the isomerization step is rate-determining and, as $[S]_0$ increases, the contribution of the term associated with k_{-1} decreases and $k_{\text{obs}} \rightarrow k_{+1}$. It is clear that at high $[S]_0$, the results in Figures 2(c) and 2(d) do not conform to eqn (6) and a different model in which both enzyme conformers are reactive is required.

Because, over the range of $[S]_0$ investigated, saturation of F by S is not observed, the model of eqn (7):

is appropriate and the associated four-parameter rate equation (eqn 8):

$$k_{\text{obs}} = \frac{k_{+1}K_{ES'}}{K_{ES'} + [S]_0} + k_{-1} + k'_{+1}[S]_0 \quad (8)$$

may be used to fit the data in Figure 2(d) over the entire range of $[S]_0$ used. These equations are analogous to the model of Scheme VI and the associated rate equation (eqn 16) discussed by Johnson [13].

The pre-steady state (acylation) phase of the papain reaction

In marked contrast with the reaction of actinidin with the thionoester substrate **1**, the corresponding reaction of papain does not exhibit the remarkable negative-dependence of k_{obs} on $[S]_0$ in any of the solvents used, including 16.7 % (v/v) acetonitrile.

There is no evidence, therefore, for a free enzyme isomerization in papain. This result demonstrates also that the unusual behaviour of actinidin is not attributable to a solvent-dependent mixing artifact. The dependences of k_{obs} on $[S]_0$ for the papain reaction in 4.3 %, 8.3 % and 16.7 % (v/v) acetonitrile are shown in Figure 3 and the characterizing parameters are compared with each other and with those for the reaction in 4.2 % (v/v) DMSO [6] in Table 1. Each of the reactions in acetonitrile, like that in DMSO, are in accord with the conventional model of eqn (2) for which equations analogous to eqns (3) and (4), with k_{-2} omitted, apply.

The other interesting characteristics of the papain reaction, in addition to the lack of evidence for a free enzyme isomerization, are (i) the fact that the values of both k_{+2} and $k_{m(\text{acyl})}$ are considerably greater in 4.3 % acetonitrile than those in 4.2 % DMSO and (ii) the unusual nature of the inhibition by acetonitrile as its content in the solvent is increased to 8.3 % and then to 16.7 %. The inhibitory effects of organic co-solvents on enzyme reactions are well known, but examples of studies involving effects of solvent on individual kinetic parameters are rare [8]. One such investigation is that reported by Clement and Bender [18] on the effect of dipolar aprotic organic solvents, including acetonitrile, on the kinetics of α -chymotrypsin-catalysed hydrolysis, where the major effect of the organic co-solvent was to increase K_m (or decrease k_{+2}/K_m) with only a small or negligible decrease in the values of k_{cat} , k_{+2} , or k_{+3} . In contrast, in the present study, the data in Table 1 demonstrate that the effect of increasing the acetonitrile content in the papain reaction is to decrease both k_{+2} and $k_{m(\text{acyl})}$ by a similar (but not identical) factor [by 54 % for k_{+2} and 35 % for $k_{m(\text{acyl})}$ on increasing the acetonitrile content from 8.3 % to 16.7 %]. These characteristics are those of mixed inhibition approximating to those of uncompetitive inhibition (eqn 9):

where I represents the inhibitor, acetonitrile.

When, as is the case for the reactions examined in the present study, k_{+3} for the deacylation of ES' can be neglected, the rate eqn (10):

$$k_{\text{obs}} = \frac{k_{+2}[S]_0/(1 + [I]/K_i)}{[S]_0 + K_{m(\text{acyl})}/(1 + [I]/K_i)} \quad (10)$$

for the model of eqn (9) is analogous to the well known steady-state rate equation for this type of inhibition (e.g. see [19]). The rate equation for uncompetitive inhibition, eqn (10), shows that, as $[I]$ is increased, k_{+2} and $K_{m(\text{acyl})}$ each decrease by the same factor. Since reversible thioimide formation by reaction of the catalytic-site thiol group of papain would probably be competitive in nature, this is unlikely to be the source of the inhibition by acetonitrile.

Concluding comment

The discovery of a free enzyme isomerization in actinidin, apparently without analogue in papain, and an unusual type of inhibition of papain by acetonitrile (i) adds to the growing body of evidence that actinidin and papain display a number of differences in active-centre behaviour and (ii) provides a striking example of the possibility of revealing additional kinetically accessible steps in enzyme catalysis by solvent perturbation. In addition, this difference might provide a possible explanation for the lack

of the binding site–catalytic site coupling in actinidin (by reaction with the F-conformer) that has such marked effects in reactions of papain with substrate-derived disulphide reactivity probes [2,3].

After this study had been completed, one of us reported another example of kinetically detectable free enzyme isomerization, observed by using a fluorescent nucleotide analogue and a subunit of the magnesium protoporphyrin IX chelatase [20]. Results such as those reported in the present paper, in [6,7,20] and in the papers from the group of Sutcliffe and Scrutton on H-transfer by quantum tunnelling (e.g. see [21]) emphasize the growing realization of the importance of protein dynamics in the understanding of enzyme mechanism.

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