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 (kobs) 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 kobs 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 μM. The unusual negative dependence of kobs 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 amid 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 thionoester 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 diastereomer 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 A185 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):

$$E + S \overset{k_1}{\rightarrow} ES \overset{k_2}{\rightarrow} ES'.P_1 \overset{k_3}{\rightarrow} (ES'.P_1)^* \overset{k_4}{\rightarrow} P_1 + (ES')^* \overset{k_5}{\rightarrow} E + P_2$$  \hspace{1cm} (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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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]1. 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 A315 (over 0.2 s for the papain reactions and over 2 s for those of actinidin) provides the observed first-order rate constant (kobs) for the formation of the dithioacyl enzymes. The rate constants were evaluated by fitting the A315/Δt data, collected by the Acorn Archimedes microcomputer (Cambridge, Cambridgeshire, U.K.), of the stopped-flow system to the equation for a single exponential process:

\[
A = P_1e^{P_2t} + P_3,
\]

where

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

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 A315, 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-Im3+ H ion pair. The dependence on [S]0 of the observed first-order rate constant, kobs, 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:

\[
E + S \rightleftharpoons ES \xrightarrow{k_2} P_1 + ES' \xrightarrow{k_3} E + P_2
\]

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
dictated by the necessity (i) to use [E] ≤ T not well-defined by the data and is probably the phase that responds markedly to variation in the composition which k

Reactions were carried out at 25 °C with [S]₀ > 10[E]; in sodium acetate buffer, pH 5.3, I 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 ±10 % of the means. The lower limit of [S]₀ = 30 µM was dictated by the necessity (i) to use [E] ≥ 3 µM 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) k₁₂/Kₘ(acl) = (1.7 ± 0.03) × 10³ M⁻¹·s⁻¹ and k₁₂ + k₁₃ = 1.95 ± 0.09 s⁻¹ from which k₁₃ = 1.85 s⁻¹; (b) k₁₂/Kₘ(acl) = (8.10 ± 0.13) × 10³ M⁻¹·s⁻¹ and k₁₂ + k₁₃ = 2.62 ± 0.04 s⁻¹ from which k₁₃ = 2.52 s⁻¹; (c) k₁₂/Kₘ(acl) = (7.90 ± 0.03) × 10³ M⁻¹·s⁻¹ and k₁₂ + k₁₃ = 2.17 ± 0.01 s⁻¹ from which k₁₃ = 2.07 s⁻¹; in (d), the line is theoretical for eqn (8) and the following parameter values: k₁₃ = 6.06 ± 1.02 s⁻¹, k₁₁ = 3.6 × 10⁻⁵ ± 1.2 s⁻¹ (this parameter is not well-defined by the data and is probably <1), K_p = (7.2 ± 1.0) × 10¹ M⁻¹·s⁻¹, Kⁱₒ = 116 ± 45 µM.

observed on the stopped-flow timescale. Exponential increase of [ES], detected by an increase in Aₐ₃₅, to its steady-state value, is followed by the rate-determining deaclylation phase over a longer timescale. The latter event involves exponential decrease in Aₐ₃₅, which is k₁₃ 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 pre-steady-state 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 ≫ [E], kobs is predicted to vary with [S]₀ according to eqn (3) [6,7,13]:

\[ k_{obs} = \frac{k_{12}[S]_0}{K_{m(acl)} + [S]_0} + k_{13} + k_{-2} \]  

(3)

where \( K_{m(acl)} = (k_{13} + k_{-2})/k_{11} \), which can approximate to \( k_{11}/k_{13} = K_c \) [14]. Eqn (3) predicts that a plot of \( k_{obs} \) against \([S]_0 \) will contain a hyperbolic component with ordinate intercept \( k_{obs} = k_{13} + k_{-2} \) when \([S]_0 = 0 \). Eqn (3) differs from the corresponding 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 \( k_{-2} + k_{13} \), whereas when irreversible formation of the acyl enzyme intermediate is assumed (eqn 2), the ordinate intercept has the value of \( k_{-2} \). For the actinidin reaction, the value of \( k_{-2} \) is small (0.1 ± 0.01 s⁻¹) and so the ordinate intercept derives largely from the value of \( k_{-2} \). Obviously, the ordinate intercept exists also for the condition \([S]_0 \ll K_{m(acl)} \), when eqn (3) becomes eqn (4):

\[ k_{obs} = \frac{k_{12}}{K_{m(acl)}} \cdot [S]_0 + k_{13} + k_{-2} \]  

(4)

Then, however, the hyperbolic component of the curve is replaced by an essentially linear response of slope \( k_{12}/K_{m(acl)} \).

Figure 2 shows the dependence of the \( k_{obs} \) on \([S]_0 \), for the pre-steady state phase of the reaction of the methyl thionoester...
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 $k_{-2}/K_{\text{m(acyl)}}$. 
approx. 1–2 × 10^4 M^{-1} s^{-1} and ordinate intercepts minus $k_{-2}$ 
(0.1 s^{-1}) (i.e. $k_{-2}$) approx. 2–3 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 mini-
mum in the region $[S]_0 < 200 \mu M$ and the existence of a minimum 
around $[S]_0 = 150 \mu M$ is demonstrated clearly in Figure 2(d) 
(16.7% acetonitrile). The decrease in $k_{obs}$ (and $k_{obs} - k_{-2}$) with 
increase in $[S]_0$ in the range 25–150 $\mu M$ 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 
cova lent intermediate and a free enzyme isomerization is eqn (5):

$$F \rightleftharpoons E \rightleftharpoons ES \rightleftharpoons ES'.P_1 \rightarrow E + P_1 + P_2$$

(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 ($k_{+2}$) 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]:

$$k_{obs} = \frac{k_{-1}K_{ES}}{K_{ES} + [S]_0} + k_{+1}$$

(6)

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

Eqn (6) shows that, as $[S]_0 \rightarrow$ zero, $k_{obs} \rightarrow k_{-1} + k_{+1}$, i.e. the 
isomerization step is rate-determining and, as $[S]_0$ increases, 
the contribution of the term associated with $k_{-1}$ decreases and $k_{obs} \rightarrow k_{+1}$. 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):

$$ES'.P_1 \rightleftharpoons E \rightleftharpoons F \rightleftharpoons FS'.P_1$$

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

$$k_{obs} = \frac{k_{-1}K_{ES}}{K_{ES} + [S]_0} + k_{+1} + k_{\rho}[S]_0$$

(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 
the thionoester substrate I, 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 $k_{\text{m(acyl)}}$ are 
comparatively rare (e.g. see [15,16]) and is characteristic 
of dipolar aprotic organic solvents, including acetonitrile, 
on the kinetics of $\alpha$-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 $k_{-2}$. 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 $k_{\text{m(acyl)}}$ by a similar (but not identical) factor [by 54% for 
$k_{+2}$ and 35% for $k_{\text{m(acyl)}}$ 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):

$$E + S \rightleftharpoons ES \rightleftharpoons I/K_i \rightarrow P_1 + ES'$$

(9)

where I represents the inhibitor, acetonitrile.

When, as is the case for the reactions examined in the 
present study, $k_{+2}$ for the deacylation of ES' can be neglected, 
the rate eqn (10):

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

(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 $K_{\text{m(acyl)}}$ 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 disulfide 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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