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THE SPECIFICITY OF ACTINIDIN AND ITS RELATIONSHIP TO THE STRUCTURE OF THE ENZYME

EDWARD N. BAKER, MICHAEL J. BOLAND, PHILIP C. CALDER and MICHAEL J. HARDMAN *

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North and Applied Biochemistry Division, D.S.I.R., Palmerston North (New Zealand)

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Summary

The kinetic parameters k_{cat}, K_m and k_{cat}/K_m, have been determined for the actinidin-catalysed hydrolyses of N-substituted amino acid esters and amides and compared to the corresponding values for papain (EC 3.4.22.2). Substrates with aromatic N-substituents have lower k_{cat}/K_m values for actinidin (EC 3.4.22.14); the difference is much smaller for substrates with aliphatic substituents. The lower k_{cat}/K_m values for actinidin generally correspond to higher K_m values suggesting that the strength of substrate binding differs between the two enzymes. This difference is explained in terms of the differences in the substrate binding sites found in X-ray crystallographic studies.

Introduction

The thiol protease, actinidin (EC 3.4.22.14), from the fruit of the Chinese gooseberry or Kiwi fruit *Actinidia chinensis*, has been shown to have many similarities to papain (EC 3.4.22.2) in kinetic behaviour and specificity [1—4]. Subsequently amino acid sequence determination [5] and X-ray crystallographic studies [6] have confirmed a general structural similarity between actinidin and papain [7]. Although their catalytic sites are virtually identical, with the same groups present in the same orientations, there are differences beyond the catalytic site in the non-polar binding pocket.

In view of the structural information now available, we decided to study

Abbreviation: pNA, *p*-nitroanilide.

* To whom reprints requests should be addressed at Massey University.

further the specificity of actinidin and to compare the kinetic parameters for interaction of substrates and inhibitors with actinidin to those for papain.

Materials and Methods

Actinidin was prepared as described previously [2], except that the extraction medium contained 25 mM cysteine instead of sodium tetrathionate and the enzyme was subsequently inactivated by tetrathionate. The enzyme concentration was determined from assays with 0.1 mM Z-Lys-ONP at pH 6.0 [2]. Papain was prepared from dried latex by the method of Baines and Brocklehurst [8].

p-Nitrophenyl hippurate was prepared by the method of Hollaway [9]. Boc-Phe-NH-CH_2CN was a generous gift from Dr. G. Lowe. All other substrates and inhibitors were obtained from Sigma, Cyclo or Vega and used without further purification.

Hydrolyses of *p*-nitrophenyl esters and the *p*-nitroanilide were followed at 348 nm ($\Delta\epsilon$ = 5400) and 410 nm ($\Delta\epsilon$ = 8800 [10]), respectively, on a Cecil CE-292 spectrophotometer. The hydrolysis of N-tosyl arginine methyl ester was followed at 246 nm ($\Delta\epsilon$ = 715 [11]) on a Cary 219 spectrophotometer. Hydrolyses of other methyl and ethyl esters were carried out in a Radiometer pH-stat assembly (see Ref. 12), using 0.01 M NaOH to maintain constant pH. Actinidin was reactivated with dithioerythritol before use [2]; a correction for spontaneous hydrolysis of the substrate was made where necessary. All reactions were carried out at 25°C in pH 6.0 phosphate buffer (I = 0.1) unless otherwise stated. K_I values of inhibitors were obtained from the effects of inhibitor concentration on k_{cat}/K_m for Z-Lys-ONP hydrolysis at pH 6.0 and 25°C [2].

Linear least-squares correlations for the Eadie plot were carried out on an IBM 1130 computer.

Results and Discussion

The steady-state rates for the actinidin-catalysed hydrolyses of various N-substituted amino acid esters and amides were measured at pH 6.0 and 25°C. Where sufficiently high substrate concentrations were attainable, the data were analysed using Eadie plots [13]; the parameters k_{cat} and K_m obtained are given in Table I. For three substrates the highest substrate concentrations obtainable were much lower than K_m and only an estimated lower limit for the K_m value is given in Table I. The corresponding values of k_{cat} and K_m for papain-catalysed hydrolyses of all the substrates are also shown, for comparison. The limited solubility of two of the substrates (Bz-Gly-ONP and Z-Gly-ONP) required the use of a higher concentration of organic co-solvent (acetonitrile) for experiments involving actinidin; the K_m values for actinidin-catalysed hydrolysis of these substrates have therefore been corrected to allow for competitive inhibition by acetonitrile, using a K_I of 1.9 M [19]. The adjustments are small (see footnotes to Table I) and reduce the difference between the K_m values for actinidin and papain.

For most substrates, K_m values are higher for actinidin than for papain

TABLE I

Kinetic constants for hydrolyses of esters and an amide, and binding of an inhibitor, by actinidin and papain.

Substrate	K_m (mM)		k_{cat} (s^{-1})	
	Actinidin [a]	Papain	Actinidin [a]	Papain
Ac-Gly-ONP [b]	>5	1.4 ± 0.2	—	2.5 ± 0.3
Bz-Gly-ONP [c]	0.31 ± 0.02	0.0117 ± 0.0003	5.8 ± 0.3	2.6 ± 0.1
Z-Gly-ONP [d]	0.12 ± 0.02	0.0093 ± 0.0010	3.4 ± 0.4	2.73 ± 0.08
Ac-Lys-OMe	78 ± 15	77 ± 25	0.7 ± 0.1	0.9 ± 0.2
Ac-Gly-Lys-OMe	>100	13 ± 2	—	1.36 ± 0.09
Z-Lys-OMe [e]	21 ± 3	2.25 ± 0.34	6.1 ± 0.4	36.2 ± 2.7
Z-Lys-ONP [f]	0.022 ± 0.002	0.0017 ± 0.0002	29 ± 2	44.5 ± 1.8
Tos-Arg-OMe	>10	1.8 ± 0.2	—	1.5 ± 0.1
Bz-Arg-OEt [g]	61 ± 12	13 ± 3	4.5 ± 0.4	16.1 ± 1.8
Bz-Arg-pNA [h]	1.8 ± 0.4	2.86 ± 0.13	0.029 ± 0.003	0.74 ± 0.03
Boc-Phe-NH-CH_2CN [i]	0.044	0.00045	—	—

[a] At pH 6.0 and 25°C.
[b] Data for papain [14].
[c] In 1.9% CH_3CN, K_m for actinidin corrected from 0.35 in 3.3% CH_3CN, data for papain [15].
[d] In 1.7% CH_3CN, K_m for actinidin corrected from 0.15 in 5% CH_3CN, data for papain [16].
[e] Data for papain [17].
[f] Data for actinidin [2], data for papain [17].
[g] Data for papain [18].
[h] Data for papain [11].
[i] K_I value for inhibition of Z-Lys-ONP (actinidin) or Z-Gly-ONP (papain) hydrolysis. Data for papain from Lowe (personal communication).

(ratios typically from 5 : 1 to 30 : 1). For the k_{cat} values no consistent trend is seen, and differences between the two enzymes are less marked. Although K_m is not necessarily a pure binding constant and may also contain contributions from kinetic terms, the significant difference in K_m for most substrates suggests that the difference between actinidin- and papain-catalysed hydrolyses lies primarily in the strength of binding. This interpretation is supported by the large difference in K_I values for binding of the inhibitor Boc-Phe-NH-CH_2CN to the two enzymes (Table I).

Non-productive binding may be important in the case of Bz-Arg-pNA, where the difference between actinidin and papain-catalysed hydrolyses lies of the value of k_{cat} rather than K_m (Table I). If the preferred mode of binding of this substrate to both enzymes is non-productive, weaker binding in the productive mode (as seen for other substrates) will result in a lower value of k_{cat} rather than a higher K_m value [20].

Because non-productive binding can be important, and because acylation is not always the rate-determining step (so that K_m contains a kinetic contribution) the most reliable measure of the specificities of actinidin and papain will be k_{cat}/K_m [21]. Values of k_{cat}/K_m were therefore calculated and are compared in Table II. Generally these were calculated from the k_{cat} and K_m values but, in those cases where the highest substrate concentrations obtainable were low relative to K_m, k_{cat} and K_m values are inaccurate, values of k_{cat}/K_m were determined from the slopes of Lineweaver-Burk plots. For all substrates with an aromatic acylamido group (including Bz-Arg-pNA), k_{cat}/K_m is considerably less

TABLE II

Comparison of k_{cat}/K_m values for actinidin- and papain-catalysed hydrolyses. Calculated from the data in Table I except as below. References are given in the footnote to Table I.

Substrate	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)		Ratio
	Actinidin	Papain	
Ac-Gly-ONP	$4.5 \cdot 10^2$ *	$1.8 \cdot 10^3$	4
Bz-Gly-ONP	$1.9 \cdot 10^4$	$2.2 \cdot 10^5$	12
Z-Gly-ONP	$2.8 \cdot 10^4$	$2.9 \cdot 10^5$	10
Ac-Lys-OMe	8.8 *	10 *	1.2
Ac-Gly-Lys-OMe	37 *	99 *	2.7
Z-Lys-OMe	$2.9 \cdot 10^2$	$1.6 \cdot 10^4$	56
Z-Lys-ONP	$1.3 \cdot 10^6$	$2.6 \cdot 10^7$	20
Tos-Arg-OMe	7 *	$7.9 \cdot 10^2$ *	110
Bz-Arg-OEt	74	$1.2 \cdot 10^3$	16
Bz-Arg-pNA	13	$2.6 \cdot 10^2$	20

* Calculated from the slopes of Lineweaver-Burk plots.

for actinidin than for papain, by a factor of between 10 and 110. This effect is observed whether the aromatic substituent is benzoyl, carbobenzoxy or tosyl. However for substrates with an aliphatic N-substituent, acetyl or acetylglycyl, the difference between the k_{cat}/K_m values for the enzymes is much smaller.

The major difference between actinidin and papain lies therefore in the interaction between aromatic N-substituents and their binding sites in the two enzymes. Such substituents probably bind in the S_2 subsite [22], which is preferentially occupied by the sidechains of Phe or Tyr residues in a polypeptide substrate [15]; interactions in the S_2 subsite make the major contribution to the specificity of papain [22].

In papain the S_2 subsite can be identified with a hydrophobic pocket formed mainly by the sidechains of Tyr 67, Pro 68, Trp 69, Phe 207, Val 133 and Val 157. Crystallographic binding studies on chloromethyl-ketone inhibitors [23] show that the aromatic substituent occupies this pocket, in Van der Waals contact (3.5—4.5 Å) with Val 133 and Val 157 and somewhat further (4.5—6.5 Å) from Tyr 67, Pro 68 and Trp 69. Model-building [15,24] and chemical modification [25] studies by Lowe are in general agreement, while emphasizing the role of Trp 69. In actinidin [6], Trp 69 is replaced by Thr and other changes are that Pro 68 becomes Ile 70, Phe 207 becomes Ser 213, and Val 133 becomes Ala 136. The major change, however, is that Ser 205, at the end of the S_2 subsite in papain, becomes Met 211, making the pocket notably shorter. If substrates bind as they apparently do in papain, a phenyl substituent would approach to within about 2.5 Å of the sidechain of Met 211. Thus, in actinidin either this sidechain must be displaced or the binding mode must differ somewhat, accounting for the observed specificity difference.

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