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Clostripain: characterization of the active site

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In view of the probability that clostripain (EC 3.4.22.8) is fundamentally different in structure from other known cysteine endopeptidases, it was of interest to examine the characteristics of the active site. Z-Phe-Lys-$CH_2S(CH_3)_2$ irreversibly and rapidly inactivated clostripain, and leupeptin was found to be the most potent reversible inhibitor yet reported for the enzyme. Clostripain was inhibited weakly by some protein inhibitors of serine endopeptidases, and required Ca^{2+} for stability and activity. Mg^{2+} and Sr^{2+} were ineffective. Rapid inactivation by diethylpyrocarbonate, reversed by hydroxylamine, indicated that histidine is essential for catalytic activity. Clostripain was more rapidly inactivated by iodoacetamide than by iodoacetate, with unique pH-dependences of reaction.

Cysteine endopeptidase; *Clostridium histolyticum*; Enzyme inhibition; Essential histidine

1. INTRODUCTION

Clostripain (EC 3.4.22.8) is a cysteine endopeptidase released into the culture medium of the anaerobic bacterium, *Clostridium histolyticum*. Structural studies of clostripain [1–3] have shown that the molecule is composed of two polypeptide chains, M_r 43000 and 15400, associated noncovalently [3]. The complete amino acid sequence has not been reported, but Cys^{41} in the M_r 43000 chain has been identified as the essential cysteine residue [4].

Most of what is known about the biochemistry of the cysteine endopeptidases has been learned from work on papain (EC 3.4.22.2) and its homologues. However, the sequence data available for clostripain indicate that this enzyme is a representative of a distinct evolutionary line. In view of this, it is of interest to compare the active site of clostripain to those of the papain-related enzymes. Accordingly, we have examined the enzyme in regard to activation by Ca^{2+}, sensitivity to reversible inhibitors, inactivation by covalently reacting inhibitors, and the pH-dependence of rates of reaction with iodoacetate and iodoacetamide.

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Abbreviations: Compound E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane; MES, 2-(*N*-morpholino)-ethanesulphonic acid; NHMec, 7-(4-methyl)coumarylamide; Tos, *p*-toluene sulphonyl; Z, benzyloxycarbonyl

2. EXPERIMENTAL

2.1. *Materials*

Clostripain was purchased from Worthington. Z-Phe-Arg-NHMec was from Bachem Feinchemikalien and diethylpyrocarbonate, leupeptin, antipain, benzamidine, aprotinin, soybean Kunitz inhibitor, soybean Bowman-Birk inhibitor and limabean trypsin inhibitor were from Sigma.

2.2. *Z-Phe-Lys-$CH_2S(CH_3)_2$, BF_4*

Z-Phe-Lys(Boc)CH_2Br was reacted with dimethyl sulphide in hot benzene as described for Z-Lys(Boc)CH_2Br [5] yielding the blocked dimethyl sulphonium bromide, m.p. 143–144.5°C (80%). The bromide was converted to the BF_4^- salt by treatment of a solution in chloroform with a small volume of saturated aqueous potassium carbonate with stirring at room temperature for 15 min. The organic layer was dried with solid potassium bicarbonate and the filtrate taken to dryness. The residue was dissolved in tetrahydrofuran (10 ml) and treated with HBF_4 (1 ml) for removal of the Boc group. After 4 h at room temperature the solvent was removed and the residue crystallized from ethanol and ether, yielding 210 mg, m.p. 156–158°C.

Analysis: Calculated for $C_{26}H_{37}N_3O_4SB_2F_8$ (661.3): C, 47.22; H, 5.64; N, 6.35; found C, 47.36; H, 5.69; N, 6.46.

2.3. *Assays*

The enzyme was preactivated in 8 mM dithiothreitol for 20 min at 0°C, and then added to the 3.0 ml reaction mixture for continuous fluorimetric assays with Z-Phe-Arg-NHMec (10 μM) as substrate at 40°C. The buffer was 20 mM Tris/HCl, containing 10 mM $CaCl_2$, 0.005% Brij 35, 2 mM dithiothreitol, pH 7.5. The fluorimeter was controlled, and data collected and analysed, by use of the FLUSYS software package [6]. Molar concentrations of active clostripain were determined by stoichiometric titration of the activity with Z-Phe-Lys-$CH_2S(CH_3)_2$. The commercial enzyme preparation was found to be 21% active, on the basis of an assay of protein [7]. Analysis by SDS/PAGE [8] showed it to be essentially homogeneous (bands of approx. 40000 and 13000 M_r), and it was used without further purification.

2.4. *Determination of K_i values*

For the determination of K_i, the rate of substrate hydrolysis in the absence of inhibitor was first recorded, and then the inhibitor was

added in a negligible volume and the new steady state was monitored. Non-linear regression analysis of the replot of fractional activity vs [I] (Enzfitter, Elsevier-Biosoft, Cambridge, England) gave K_i. Control experiments showed that the condition $[S] \ll K_m$ was met, so that no correction for the effect of substrate on observed K_i was needed.

2.5. *Rate constants for irreversible inactivation*

Rates of inactivation were measured under pseudo-first-order conditions, with [I] ≫ [E]. For measurements with iodoacetate and iodoacetamide, the enzyme was preactivated in 8 mM dithiothreitol, and then diluted so that the final reaction mixture contained only 90 μM dithiothreitol, a concentration that was far below that of the alkylating agent. The reactions were carried out at 25°C in 0.1 M sodium acetate (pH 4.92–5.58), MES (pH 5.39–7.25) or Tris/HCl (pH 7.05–9.03) buffer. The concentrations of reactants were varied with pH, to facilitate the accurate measurement of rates, in the range 0.17–1.75 nM for clostripain, 1–5 mM for iodoacetate, and 0.3–1 mM for iodoacetamide. Apparent rate constants (K_{obs}) for inactivation were found by non-linear regression analysis of the apparent pseudo-first-order curves of inactivation [6,9]. $k_{2(app)}$ was calculated as $K_{obs}/[I]$.

3. RESULTS AND DISCUSSION

3.1. *Activation by Ca^{2+}*

Clostripain was dissolved in 10 mM EDTA, and dialysed against 50 mM Tris/HCl buffer, pH 7.5, for 24 h. The enzyme then showed no activity in an assay without Ca^{2+}. With 10 mM Ca^{2+}, it progressively regained just 27% of the original activity, the process being complete within 10 min. Below 1 mM Ca^{2+}, the amount of activity regained was dependent upon $[Ca^{2+}]$, with half-maximal activity being produced by 0.195 mM Ca^{2+}. Mg^{2+} and Sr^{2+} caused no activation at 10 mM or lower concentrations.

We conclude that Ca^{2+} stabilizes clostripain, confirming the early work of Labouesse and Gros [10]. Ca^{2+} also activates clostripain in a rather slow process with half-maximal activity being achieved at a metal ion concentration of about 0.2 mM. This affinity is of an order similar to that of some forms of calpain [11].

3.2. *Reversible inhibition*

Table I shows the K_i values for reversible inhibition of clostripain. The most potent inhibitors were the peptide aldehydes, leupeptin and antipain, both of which contain the arginine sidechain in P1 [12]; chymostatin, which lacks this, was bound much more weakly. Protein inhibitors of some serine endopeptidases, with either lysine (soybean Kunitz inhibitor, aprotinin, lima bean trypsin inhibitor) or arginine (soybean Bowman-Birm inhibitor) in P1 were significant inhibitors of clostripain, although the K_i values were much higher than for trypsin. Unlike its behaviour towards substrates, clostripain did not appear to discriminate in favour of arginine over lysine in this series of inhibitory proteins. Chicken cystatin, a tight binding protein inhibitor of papain and related cysteine endopeptidases [13], inhibited clostripain weakly.

Table I

K_i values for reversible inhibitors of clostripain

	K_i (μM)
Leupeptin	0.37
Antipain	0.58
Chymostatin	9.2
Compound E-64	17
Benzamidine	240
Soybean Kunitz inhibitor	25
Aprotinin	28
Soybean Bowman-Birk inhibitor	>200
Limabean trypsin inhibitor	290
Chicken cystatin	≫20

Inhibition constants were determined at pH 7.5, 40°C, as described in section 2.

3.3. *Inactivation of clostripain by diethylpyrocarbonate*

Histidine contributes to the catalytic mechanism of all endopeptidases of cysteine, serine and metallo-type that have so far been thoroughly characterized, but no direct evidence has yet been provided of a role for histidine in clostripain. Experiments were therefore made with a specific modifier of histidine, diethylpyrocarbonate [14].

Diethylpyrocarbonate (as a 100 mM ethanolic solution) was introduced to a final concentration of 330 μM into a continuous assay of clostripain (0.174 nM) at 40°C in 0.1 M Tris-HCl buffer, pH 7.5, containing 10 mM Ca^{2+} and 0.1 mM dithiothreitol. There was an essentially instantaneous, complete loss of activity. Introduction of hydroxylamine hydrochloride into the cuvette (to 6.6 mM) resulted in the rapid recovery of 85% of the original activity (not shown). Control experiments confirmed that there was no inhibition by ethanol alone, or activation by hydroxylamine in the absence of diethylpyrocarbonate.

These results indicate that one or more histidine residues are essential for the activity of clostripain [14]. Diethylpyrocarbonate is known to react non-specifically with cysteine residues, but extremely slowly, and the adduct is stable to hydroxylamine [15].

Table II

Second order rate constants for inactivation of clostripain by covalently reacting reagents

Compound	Rate constant ($M^{-1} \cdot s^{-1}$)	Reference
Tos-Phe-CH_2Cl	46.7	[24]
Phe-Ala-Lys-Arg-CH_2Cl	1.86×10^6	[25]
Phe-Ala-Lys-Arg-CH_2CH_2Cl	1.01×10^7	[25]
D-Phe-Pro-Arg-CH_2Cl	4.5×10^4	[25]
D-Phe-Pro-Arg-CH_2CH_2Cl	1.4×10^6	[25]
Z-Phe-Arg-CHN_2	8.6×10^4	[26]
Tos-Lys-CH_2Cl	8.7×10^4	[24]
Z-Lys-$CH_2S^+(CH_3)_2$	1.66×10^5	[5]
Z-Lys-$CH_2S^+(CH_3)CH_2Ph$	1.07×10^5	[5]
Z-Phe-Lys-$CH_2S^+(CH_2)_2$	1.4×10^6	Present study

3.4. *Other covalently reacting inactivators of clostripain*

The rate constant for inactivation of clostripain by Z-Phe-Lys-$CH_2S(CH_3)_2$ was found to be 1.4×10^4 $M^{-1} \cdot s^{-1}$, distinctly higher than the values reported for two similar sulphonium salts lacking the additional phenylalanine (Table II).

Compound E-64, which inactivates most cysteine endopeptidases rapidly and irreversibly [16], was found to give only reversible inhibition of clostripain (K_i 17 μM). It may well be that the arginine-like sidechain of E-64, which binds in P2 of papain [17] is bound by clostripain in a mode that is unproductive for any covalent reaction. Also, in view of the low reactivity of iodoacetate with clostripain (see below), we speculate that the carboxyl group of the *trans*-epoxysuccinyl moiety of E-64, which facilitates the reaction with papain by an interaction with the active site imidazolium ring [17], would not similarly favour the reaction with clostripain.

The rate constants for inactivation of clostripain by iodoacetate and iodoacetamide were determined at pH 7.5 and 25°C. The results are presented in Table III, in comparison to literature values for those for several other cysteine endopeptidases obtained under slightly different conditions.

Papain and most of its homologues react much more rapidly at acidic and neutral pH with iodoacetate than with iodoacetamide [18]. Two members of the papain superfamily, stem bromelain and glycyl endopeptidase (previously known as papaya proteinase IV) are unusual in their slow reactions with iodoacetate [19,20], but even for these the reaction is faster than for iodoacetamide. The enhanced rate with iodoacetate has been attributed to favourable alignment of the carboxylate of the alkylating agent with the imidazolium ion of the active site histidine [18]. In contrast, clostripain reacted about 6-fold faster with iodoacetamide than with iodoacetate. This behaviour is reminiscent of that of simple thiols [18], thiolsubtilisin (the derivative of subtilisin in which the active site serine has been replaced by cysteine: [21]), and of a cysteine endopeptidase from germinating bean seeds [22]. Such behaviour suggests that a thiolate-imidazolium ion pair exactly like that of papain does not exist in clostripain, and is not obligatory for effective catalysis by a cysteine-type endopeptidase.

Table III

Rate constants for inactivation of clostripain by iodoacetate and iodoacetamide, compared with literature values for other cysteine endopeptidases

	Iodo-acetate	Iodo-acetamide	Ratio[a]
Bean endopeptidase (pH 6.5, 25°C) [22]	0.11	20	181.82
Thiolsubtilisin (pH 7.5, 25°C) [21]	0.84	6.3	7.5
Clostripain (pH 7.5, 25°C)	0.79	5.2	6.58
Stem bromelain (pH 6.8, 40°C) [19]	17.7	5.5	0.31
Glycyl endopeptidase (pH 6.8, 30°C) [20]	2.2	0.5	0.23
Papain (pH 6.8, 30°C) [20]	1000	46	0.05

[a] Calculated as the rate constant for inactivation with iodoacetamide divided by that with iodoacetate

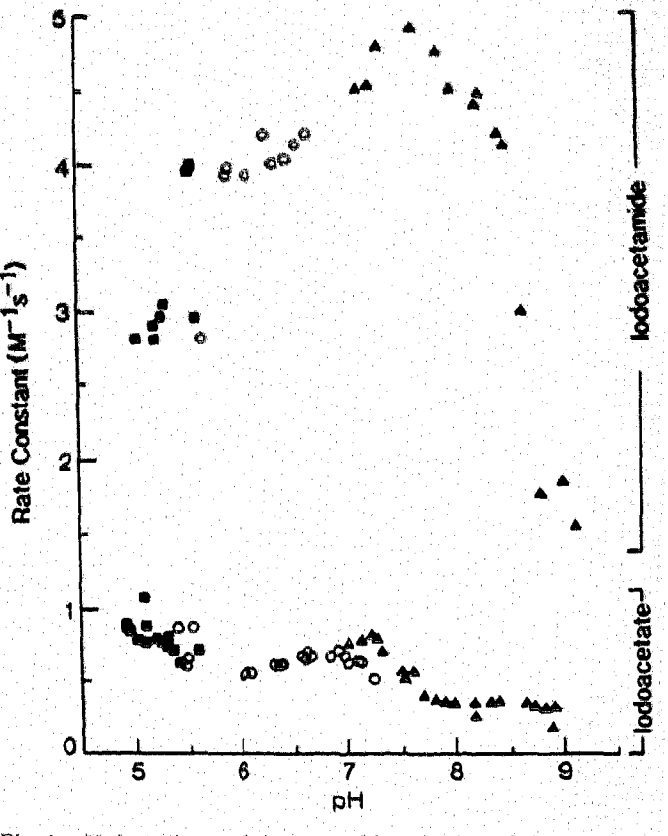

Fig. 1. pH-dependence of the rates of inactivation of clostripain by iodoacetamide and iodoacetate. Apparent pseudo-first-order curves of inactivation were obtained in the presence of 90 μM dithiothreitol at 25°C as described in section 2. The rate constants for inactivation were found by non-linear regression analysis, giving K_{obs}. $k_{2(app)}$ was calculated as $K_{obs}/[I]$. The experiments were performed in: (■) acetate; (○) MES; (▲) Tris, buffers.

3.5. *pH dependence of the reaction of clostripain with iodoacetate and iodoacetamide*

The profiles for pH dependence of the rate constants for inactivation of clostripain by iodoacetate and iodoacetamide are shown in Fig. 1. An asymmetric bell-shaped profile was obtained with iodoacetamide, with a maximum at pH 7.5. (The enzyme was unstable below pH 5 and above pH 9.) In contrast, papain has been shown to exhibit a double sigmoid curve with two pK values of 4.05 and 8.5 [23].

The profile for inactivation of clostripain by iodoacetate was more complex, indicating the existence of two reactive species associated with the active site. There was a bell-shaped profile in the range pH 6–8,

with a maximum near pH 7. At lower pH values, the rate of alkylation increased, giving another rate maximum at pH 5. Such a pH rate profile has not been observed previously for any cysteine endopeptidase. The pH dependence of alkylation of papain with iodoacetate is a bell-shaped curve, with a rate maximum at pH 6.0, governed by pK values of 3.6 and 8.3. Cathepsin B has an unusual profile, with a single rate maximum at acidic pH (about 4) controlled largely by a group with pK 5.5 [23].

The unique nature of the pH-rate curves of clostripain with the two alkylating agents is evidence that the ionic state of the active site is governed by groups for which there are no parallels in the other cysteine endopeptidases that have been studied.

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