J. Enzyme Inhibition, 1990, Vol. 4, pp. 35–42
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PEPTIDE DIAZOMETHYL KETONES ARE INHIBITORS OF SUBTILISIN-TYPE SERINE PROTEASES

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(Received 5 September 1989)

Peptide diazomethyl ketones, well known as specific cysteine protease inhibitors are also potent inhibitors of the microbial serine proteases thermitase (EC 3.4.21.14) and subtilisin Carlsberg (EC 3.4.21.14). The affinity of the enzymes towards the synthetic inhibitors Z-Ala$_n$-PheCHN$_2$ (n = 0, 1, 2) depends on the chain length and is in the same range as for the corresponding chloromethyl ketones. Both kinds of inhibitors react irreversibly in a 1:1 ratio with the enzymes and covalently bind to the active site histidine of both subtilisin Carlsberg and thermitase despite the fact that thermitase contains an active-site cysteinyl residue. The mechanism of the inhibition reaction is discussed.

KEY WORDS: Serine proteases, thermitase, subtilisin Carlsberg, peptide diazomethyl ketones, irreversible inhibition.

INTRODUCTION

Peptide diazomethyl ketones have been described chiefly as selective inhibitors of cysteine proteases.[1,2] This inactivation is due to covalent modification of the cysteine residue in the active site and is irreversibly.[3,4] However, in contrast with the generally assumed inertness of serine proteases to peptide diazomethyl ketones, plasma kallikrein[5] as well as post-proline-cleaving enzyme from macrophages[6] were inactivated by this group of inhibitors although with considerably lower affinity.

In this context it should be of interest to investigate the reactivity of diazomethyl ketones with subtilisin-type serine proteases, especially with enzymes containing one cysteine residue.[7] To this group of serine proteases belongs the microbial enzyme thermitase from *Thermoactinomyces vulgaris*.[8] Thermitase contains a cysteinyl residue in position 64 of the primary structure which means that this residue is in the immediate vicinity of the active site triad.[9] Modification of this cysteinyl residue with

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Abbreviations: Z-, benzyloxycarbonyl; Suc-, succinyl; -pNA, 4-nitroanilide; CHN$_2$-, diazomethyl ketone; CH$_2$Cl-, chloromethyl ketone; CH$_3$-, methylketone; DMF, dimethylformamide; DMSO, dimethylsulfoxide; CM-His, 3-carboxymethyl histidine; CM-Cys, carboxymethyl cysteine.

Hg^{2+} is associated with a complete loss of activity.[10] Furthermore peptide methyl ketones and chloromethyl ketones show a much higher affinity to thermitase in contrast to other cysteine-free serine proteases[11,12] such as subtilisin Carlsberg which belongs to the same family of microbial enzymes and shows 44% sequence homology compared with thermitase.[9]

Therefore we investigated the inhibition kinetics of thermitase and subtilisin Carlsberg using peptide diazomethyl ketones of the general formula Z-Ala_n-$PheCHN_2$ (n = 0, 1, 2). Amino acid analysis of the enzymes covalently modified with the tripeptide diazoketone Z-Ala_2-$PheCHN_2$ should give information about the labelled amino acid. Also additionally for thermitase the SH-content of the native and modified enzyme was determined. The findings were compared with previous results from modification experiments with the radiolabelled chloromethyl ketone Z-Ala_2-$Phe^{14}CH_2Cl$.

MATERIALS AND METHODS

Enzymes

Lyophilized thermitase was prepared by Dr. W.E. Höhne (Institute of Biochemistry, Humboldt University Berlin) according to Frömmel *et al.*.[13] Lyophilized subtilisin Carlsberg was an authentical sample from the Carlsberg Laboratories, Copenhagen (Denmark). The protein concentrations were estimated by use of the specific absorptions $A_{280}^{1\%}$ = 17.0 for thermitase and $A_{280}^{1\%}$ = 9.6 for subtilisin Carlsberg.[14]

Substrate

Suc-Ala_2-PhepNA was prepared according to Brömme and Fittkau.[15]

Inhibitor Synthesis

The benzyloxycarbonyl-protected compounds Z-Ala_n-PheOH (n = 0, 1, 2) were activated by use of the mixed anhydride method and then converted into the diazomethyl ketones with diazomethane.[16,17] The crude products were recrystallized using common solvents, resulting in pale yellow crystals. The homogeneity of the diazomethyl ketones was checked by thin layer chromatography in several solvent systems. Optical activity was determined with the POLAMAT A (Carl Zeiss Jena, GDR) with c = 1 in methanol.

Z-$PheCHN_2$: mp = 82–85°C (from ether/petroleum ether); $[\alpha]_D^{25}$ = −38.4°

Z-Ala-$PheCHN_2$: mp = 118–122°C (from ethyl acetate/petroleum ether); $[\alpha]_D^{25}$ = −51.6°

Z-Ala_2-$PheCHN_2$: mp = 142–143°C (from ethyl acetate/ether); $[\alpha]_D^{25}$ = −62.5°

Peptide diazomethyl ketones could be stored at −20°C under vacuum for 4 weeks without any degradation.

3-Carboxymethyl histidine was synthesized according to a method of Banaszak and Gurd[18] as modified by Paetzold.[19] Carboxymethyl cysteine was a gift from Prof. Dr. Hermann (Institute of Biochemistry, Martin-Luther-University, Halle).

Micropreparative Scale Modification Experiments

The peptide diazomethyl ketones were freshly dissolved in dimethylformamide and then diluted with 10 mM Tris-HCl buffer, pH 7.2, and finally mixed with the enzyme dissolved in the same buffer to give the concentrations shown in Table I. The modification mixture was incubated at room temperature in the dark until a residual activity of less than 5% was reached. The modified proteins were separated from the excess of inhibitor by ultrafiltration (Amicon, Netherlands; membrane YM 10) and then lyophilized.

Kinetic Studies

The kinetic experiments were carried out in 0.1 M Tris-HCl buffer, pH 8.2, at 25°C using a thermostated recording Eppendorf-photometer. To follow the cleavage of the substrate Suc-Ala_2-PhepNA (initial concentration of 1.0×10^{-3} M) the formation of 4-nitroanilide was measured at 405 nm ($\varepsilon = 9800\,M^{-1}cm^{-1}$).

The K_i and k_2 values were determined according to the method of Kitz and Wilson.[20] For the reaction of thermitase with Z-Ala_2-$PheCHN_2$ the K_i value was estimated by use of the φ-kinetic[11] since the initial concentrations of enzyme and inhibitor are of the same order of magnitude. In all other cases the initial concentrations of the inhibitors in the reaction mixture were in the range of the expected inhibition constant K_i; the enzyme concentration was 1.4×10^{-8} M and 6.5×10^{-8} M for thermitase and subtilisin Carlsberg, respectively.

Five to six concentrations of inhibitor and five to seven incubation times (15 to 90 s for higher inhibitor concentrations, and a maximum of 480 s for lower inhibitor concentrations) were used. All parameters given in Table II are averaged values for two or more parallel measurements.

The stock solutions of the inhibitors were freshly prepared daily since light accelerates the decay of the peptide diazomethyl ketones.

Determination of the Thiol Groups

The quantitative determination of the thiol groups was carried out according to the DTNB-method of Ellman[21] as slightly modified by Hansen[22] and Frömmel[13] on a double-beam spectrophotometer Shimadzu-Sapcom-1A. Before SH-titration the protein, native as well as modified, was denaturated with 10% trichloric acid.

Amino Acid Analysis

The amino acid analysis of the native and modified enzymes were performed after

TABLE I
Experimental conditions for enzyme modification on the micropreparative scale

Enzyme	(μM)	Inhibitor	(mM)	amount DMF (v/v %)	incubation time (h)
thermitase	17	Z-$PheCHN_2$	0.83	10	2
thermitase	10	Z-Ala_2-$PheCHN_2$	1.0	5	1
subtilisin Carlsberg	10	Z-Ala_2-$PheCHN_2$	1.5	10	4

TABLE II

Kinetic constants for the reaction of thermitase and subtilisin Carlsberg with peptide diazomethyl ketones and corresponding chloromethyl ketones

	Thermitase			subtilisin Carlsberg		
	K_i(M)	$k_2(s^{-1})$	k_2/K_i ($M^{-1} \times s^{-1}$)	K_i(M)	$k_2(s^{-1})$	k_2/K_i ($M^{-1} \times s^{-1}$)
Z-PheCHN$_2$	5.3×10^{-4}	2.5×10^{-3}	5	$> 1 \times 10^{-3}$	n.d.	n.d.
Z-Ala-PheCHN$_2$	6.8×10^{-5}	4.5×10^{-2}	662	7.5×10^{-4}	3.4×10^{-3}	5
Z-Ala-Ala-PheCHN$_2$	1.4×10^{-7}†	3.7×10^{-2}*	> 260 000	2.7×10^{-5}	2.0×10^{-2}	741
Z-PheCH$_2$Cl	1.4×10^{-5}	1.5×10^{-3}	107[Ref.15]	$> 1 \times 10^{-4}$	n.d.	n.d.
Z-Ala-PheCH$_2$Cl	1.5×10^{-6}	1.5×10^{-3}	1 000[Ref.11]	7.8×10^{-5}	1.8×10^{-3}	23
Z-Ala-Ala-PheCH$_2$Cl	7.4×10^{-8}	2.3×10^{-3}	31 000[Ref.15]	7.8×10^{-7}	2.0×10^{-3}	2 560

†determined with the φ-kinetics[11]

*$k_2 \approx k_{obs}$ with [I] = 7.9×10^{-7} M.

performic acid oxydation, lyophilization of the products and total hydrolysis with 6 N HCl for 24 h. The amino acid analyzer Biotronik model 600 was calibrated with carboxymethyl cysteine and 3-carboxymethyl histidine.

RESULTS

Both enzymes, thermitase and subtilisin Carlsberg were inhibited in an irreversible manner by peptide diazomethyl ketones. The kinetic parameters given in Table II express a higher affinity of the inhibitors for thermitase in comparison to subtilisin Carlsberg. For both enzymes, with an increasing number of amino acids of the peptide chain of the diazomethyl ketones the inhibition constant K_i decreases. This fact together with an associated higher rate constant k_2 leads to significant increase in the second order rate constant k_2/K_i for both enzymes. The K_i values of the peptide diazomethyl ketones are about one order of magnitude greater than the inhibition constants of the chloromethyl ketones with a comparable amino acid sequence. Remarkable is the general higher inactivation velocity, expressed as k_2 values, of the peptide diazomethyl ketones by comparison with the corresponding chloromethyl ketones. When the incubation time was prolonged for a period of up to one hour in order to get a completely inactivated enzyme on a micropreparative scale a considerable deviation from the initial first order kinetics was observed (Figure 1). Similar activity-time curves have also been described by Green and Shaw[23] and may be due to an enzymatic cleavage of the inhibitor molecule in competition with the inhibition

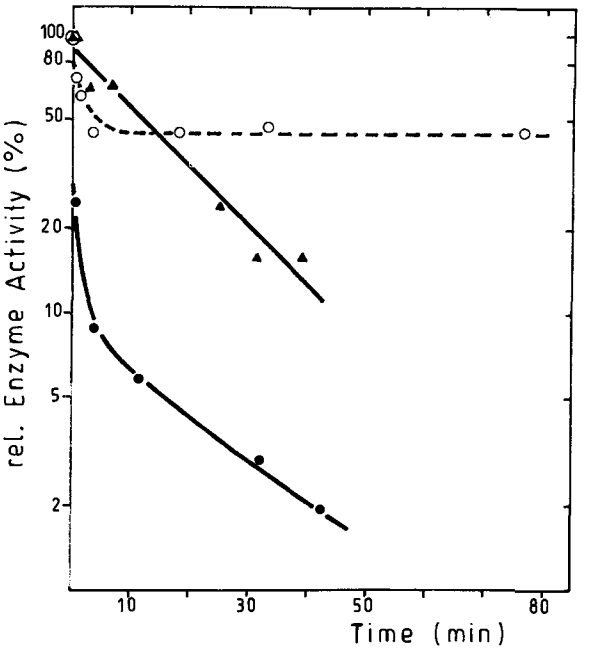

FIGURE 1 Time dependence of the inactivation of thermitase by Z-PheCHN$_2$ (▲, molar ratio enzyme/inhibitor 1:50) and Z-Ala$_2$-PheCHN$_2$ (○, molar ratio 1:50; ●, molar ratio 1:100).

TABLE III
Comparison of the SH content of native and diazomethyl ketone-modified thermitase as estimated by the method of Ellman[21]

thermitase	inhibitor	mol SH group mol protein	relative amount of SH
native	–	0.95	100%
modified	Z-PheCHN$_2$	0.92	97%
modified	Z-Ala$_2$-PheCHN$_2$	0.90	95%

reaction. The destruction of peptide diazomethyl ketones by splitting of the inhibitor to the peptide acid, probably with liberation of diazomethane, has been discussed[5]. Despite the slow inactivation process, modified enzymes with residual activity from 1–5% were obtained. This small residual activity persisted after ultrafiltration and lyophilization of the samples and is additional evidence for an irreversible type of inhibition.

Using Ellman's reagent to titrate the thiol group in thermitase inhibited by Z-PheCHN$_2$ and Z-Ala$_2$-PheCHN$_2$, respectively, it was shown that the modified enzyme in both cases possessed a free SH-group like the unmodified enzyme (Table III). This result was initial evidence that the covalent bond formed with the diazomethyl ketone inhibitors does not occur via the single cysteinyl residue of thermitase. Amino acid analysis of thermitase and subtilisin Carlsberg, modified by the tripeptide inhibitor Z-Ala$_2$-PheCHN$_2$ shows that in both cases one histidyl residue is modified by the diazoketone and a single 3-carboxymethyl histidine residue is formed per protein molecule (Table IV). In the hydrolysate of modified thermitase 3-carboxymethyl cysteine could not be detected. This means that not only in subtilisin Carlsberg but also in thermitase the active site histidine (and not cysteine in the latter case) is modified. As expected in the modified proteases the amount of alanine increases by two residues in comparison with the native enzymes due to the presence of the peptide inhibitor in the modified enzymes. The results of the complete amino acid analysis are in acceptable agreement with the theoretical values with a very good separation of the peaks for CM-His and the other amino acids.

The stoichiometric interaction between the Z-tripeptide diazomethyl ketone and the enzymes in a 1:1 ratio was also found in experiments carried out to modify

TABLE IV
Amino acid analysis of native and Z-Ala$_2$-PheCHN$_2$ modified thermitase and subtilisin Carlsberg

	thermitase		subtilisin Carlsberg	
	native	modified	native	modified
CM-Cys	0.00 (0)[a]	0.00 (0)[b]	(0)[a]	(0)[b]
CM-His	0.00 (0)	0.98 (1)	0.00 (0)[a]	1.17 (1)[b]
Cys[c]	0.94 (1)	0.90 (1)	(0)	(0)
His	4.41 (4)	d. (3)	5.33 (5)	d. (4)
Ala	44.29 (44)	46.21 (46)	40.60 (41)	42.10 (43)
Phe	3.00 (3)	3.17	3.86 (4)	4.43

[a]calculated from the amino acid sequence.
[b]expected amino acid content.
[c]Cys, as cysteic acid after performic acid oxidation.
[d]not determined due to overlapping products formed from the hydrolyzed inhibitor-Phe residue.

thermitase with the radiolabelled inhibitor[19,24] Z-Ala_2-Phe$^{14}CH_2Cl$. In the tryptic hydrolysate of the labelled enzyme, radioactivity was only found in the SH-containing peptide. This peptide could be easily isolated using thiopropyl-sepharose. The analysis of the total hydrolysate of this SH-peptide by two-dimensional high voltage electrophoresis and paper chromatography as well as by routine amino acid analysis showed the formation of radioactive 3-carboxymethyl histidine.

DISCUSSION

The results of our investigations clearly demonstrate that peptide diazomethyl ketones in addition to the known inhibition of the two serine proteases plasma kallikrein[5] and post-proline-cleaving enzyme from macrophages[6] also inactivate two other serine proteases, the microbial enzymes thermitase and subtilisin Carlsberg. This finding leads to the assumption that other hitherto uninvestigated serine proteases may be inhibited by peptide diazomethyl ketones. Therefore this class of compounds cannot be descibed, as previously, as specific cysteine protease inhibitors.

The inactivation reaction possesses a stoichiometry of one-to-one and is irreversible. Peptide diazomethyl ketones as well as the corresponding chloromethyl ketones alkylate the imidiazole of the active site histidine resulting in the presence of 3-carboxymethyl histidine in the total acid hydrolysate of the modified enzymes.

Covalent bonding of the diazoketones to the active site cysteine of thermitase can be excluded: only 3-carboxymethyl histidine is formed and carboxymethyl cysteine could not be detected. Furthermore in the modified thermitase one free thiol group was found. These facts demonstrate unambiguously when taken with other investigations[11] that thermitase should be classified as a serine protease and not as a cysteine protease.

Serine proteases react with peptide substrates and inhibitors initially by formation of a tetrahedral complex.[25] Starting with this intermediate (I) we propose the following steps in the reaction mechanism of serine proteases with diazomethyl ketones (Figure 2). In this intermediate the diazomethyl-C-atom is protonated by the im-

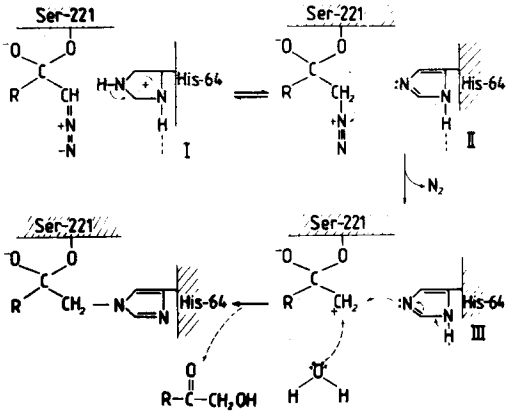

FIGURE 2 Mechanism of labelling of the active site histidine in serine proteases by diazomethyl ketones. Numbering of the active site amino acids follows the sequence of thermitase[9].

idazolium residue of the histidine (II). Nucleophilic attack of the C_1-atom of the diazonium ion by the imidazole nitrogen then occurs simultaneously with elimination of N_2 resulting in alkylation of the histidine (III). On the other hand our kinetic results point to a reaction with two succeeding steps: firstly the formation of a carbonium ion by liberation of nitrogen from the diazonium ion and secondly the alkylation of the imidazole nitrogen by the carbonium-carbon atom. As a competitive reaction to the second step the carbonium ion may also be attacked by other nucleophiles i.e. water. The forming of such hydroxymethyl ketones has been reported by Watanabe *et al.* for the reaction of Z-PheCHN$_2$ with chymotrypsin.[2] The existence of an intermediary carbonium ion and the possibility of side reactions would explain the higher rate constants (k_2) for diazomethyl ketones compared with chloromethyl ketones and the deviation of the kinetics from first order in our experiments.

References

1. Shaw, E. and Green, G.D.J. (1981) *Meth. Enzymol.*, **80**, 820
2. Watanabe, H., Green, G.D.J. and Shaw, E. (1979) *Biochem. Biophys. Res. Commun.*, **89**, 1354
3. Leary, R., Larsen, D., Watanabe, H. and Shaw, E. (1977) *Biochemistry*, **16**, 5857
4. Brocklehurst, K. and Malthouse, P. (1978) *Biochem. J.*, **175**, 761
5. Zumbrunn, A., Stone, S. and Shaw, E. (1988). *Biochem. J.*, **250**, 621
6. Green, G.D.J. and Shaw, E. (1983) *Archs. Biochem. Biophys.*, **25**, 1331
7. Baudyš, M., Meloun, B.,Kostka, V., Hausdorf, G., Frömmel, C. and Höhne, W.E. (1987) In: J. Chaloupka and V. Krumphanzel (Eds.), Proceedings of the Symposium *Extracellular Enzymes of Microorganisms*, pp. 59–71, New York, Plenum Press.
8. Kleine, R. and Kettmann, U. (1982) *Hoppe-Seyler's Z. Physiol. Chem.*, **363**, 843.
9. Meloun, B., Baudyš, M., Kostka, V., Hausdorf, G., Frömmel, C. and Höhne, W.E. (1985) *FEBS Letters*, **183**, 195
10. Hansen, G., Frömmel, C., Hausdorf, G. and Bauer, S. (1982) *Acta Biol. Med. Germ.*, **41**, 137.
11. Fittkau, S., Smalla, K. and Pauli. D. (1984) *Biomed. Biochim. Acta*, **43**, 887.
12. Brömme, D., Bartels, B., Peters, K., Kirschke, H. and Fittkau, S. (1988) In: B. Penke and A. Török (Eds.), *Peptides* pp. 53–56, Berlin, New York, Walter de Gruyter.
13. Frömmel, C., Hausdorf, G., Höhne, W.E., Behnke, U. and Ruttloff, H. (1978) *Acta Biol. Med. Germ.*, **37**, 1193.
14. Ottesen, M. and Svendsen, I. (1970) *Meth. Enzymol.*, **19**, 199.
15. Brömme, D. and Fittkau, S. (1985) *Biomed. Biochim. Acta*, **44**, 1089.
16. Fittkau, S. (1973) *J. Prakt. Chemie*, **315**, 1037.
17. Jahreis, G., Smalla, K. and Fittkau, S. (1984) *J. Prakt. Chemie*, **326**, 41.
18. Banaszak, L.J. and Gurd, F.R.N. (1964) *J. Biol. Chem.*, **239**, 1836.
19. Paetzold, G. (1982) Diplomarbeit, Martin-Luther-University Halle-Wittenberg, GDR.
20. Kitz, R. and Wilson, I.B. (1962) *J. Biol. Chem.*, **237**, 3245.
21. Ellman, G. (1958) *Archs. Biochem. Biophys.*, **74**, 443.
22. Hansen, G., Eifler, R. and Heitmann, R. (1972) *Acta Biol. Med. Germ.*, **23**, 977.
23. Green, G.D.J. and Shaw, E. (1981) *J. Biol. Chem.*, **256**, 1923.
24. Baudyš, M., Kostka, V., Hausdorf, G., Fittkau, S. and Höhne, W.E. (1983) *Int. J. Pep. Prot. Res.*, **22**, 66.
25. Polgár, L. and Asbóth, B. (1986) *J. Theoret. Biol.*, **121**, 323.