INHIBITION OF *ACHROMOBACTER* COLLAGENASE BY BROMOACETONE AND BY ZINC IONS

P. HERRY and V. KEIL-DLOUHA

Unité de Chimie des Protéines, Institut Pasteur 28, rue du Docteur Roux, 75724 Paris Cedex 15, France

Received 29 August 1978

1. Introduction

The collagenase from *Achromobacter iophagus* is an extracellular Zn-metalloproteinase (EC 3.4.24.8) [1,2] which splits preferentially the bonds X$^{\downarrow}$–Gly–Pro and X$^{\downarrow}$–Gly–Ala in the helical regions of collagen [3]. In bacterial culture its synthesis is induced by collagen or by its high molecular weight fragments [4]. Recent results obtained in our laboratory [5] have shown that the active form of *Achromobacter* collagenase (spec. act. 2 μkat/mg) is a dimer of 70 000 daltons which can be dissociated into two polypeptide chain subunits of 35 000 daltons. A unique N-terminal sequence determined in the dimer indicates that the two subunits are identical at least in their N-terminal regions. The dissociation under described conditions is irreversible and brings about the loss of enzymatic activity.

Achromobacter collagenase resembles in its amino acid composition, molecular weight of the subunit, as well as in the metal dependence, two bacterial metalloproteinases, thermolysin and the neutral proteinase from *Bacillus subtilis*. For practically identical molecular weights (35 000 and 34 300), *Achromobacter* collagenase (subunit) and thermolysin have a very similar content of basic amino acids: 9 and 10 arginines, 10 and 11 lysines and both contain 8 histidines.

In both thermolysin and *B. subtilis* proteinase a single histidine residue has been shown indispensable for the catalytic activity and both are inhibited by an excess of Zn^{2+} [6].

Abbreviations: PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetate; PZ–Pro–Leu–Gly–Pro–D-Arg, 4-phenylazobenzoyl-oxycarbonyl–L-prolyl–L-leucyl–glycyl–L-prolyl–D-arginine dihydrate

This study presents evidence for the importance of a histidine residue for the activity of *Achromobacter* collagenase. The treatment of collagenase with bromoacetone, at pH 6.5, leads to the loss of its proteolytic activity accompanied by the disappearance of one histidyl residue. The competitive inhibitor prolyl–leucyl–sarcosyl–proline protects the enzyme from this modification. On the other hand, the dissociation of the enzyme into subunits increases the accessibility of the histidine residues to the reagent. Like thermolysin and *B. subtilis* neutral protease, *Achromobacter* collagenase was found to be inhibited by zinc ions. However this inhibition is without influence on the accessibility of histidine residues to bromoacetone.

2. Materials and methods

2.1. *Materials*

Crude collagenase from *Achromobacter iophagus* of spec. act. 0.3 μkat/mg was purchased from Institut Pasteur Production. Homogeneous *Achromobacter* collagenase of mol. wt 70 000 and spec. act. 2.0 μkat/mg, was obtained by chromatography on DE-32 cellulose and Sephadex G-100 as in [2,7].

2.2. *Enzyme assay and analytical methods*

Collagenase activity was measured colorimetrically using 4-phenylazobenzoyl-oxycarbonyl–L-prolyl–L-leucyl–glycyl–L-prolyl–D-arginine dihydrate (Fluka) according to [8]. Numerical data have been recalculated on the basis of 1 nkat = 90 units.

Protein concentrations were determined by the Lowry method [9]. Polyacrylamide gel electro-

phoresis (PAGE) was carried out according to [10] in 25 mM Tris–0.2 M glycine buffer (pH 8.3) which was 0.1% with respect to sodium dodecylsulfate. Phosphorylase *b* (93 000), bovine serum albumin (68 000), aldolase (subunit 40 000) and carbonic anhydrase (29 000) were used as standard markers. The proteins were heated for 5 min at 100°C in 1% β-mercaptoethanol containing 3% sodium dodecylsulfate prior to electrophoresis. Amino acid analyses were performed with a Beckman Multichrome amino acid analyzer. Protein hydrolyses were carried out at 110°C under nitrogen. The cysteine and methionine content was obtained after oxidation with performic acid [11]. Tryptophan was determined after hydrolysis by methane–sulphonic acid [12].

2.3. *Reaction with bromoacetone*

Reaction of collagenase with bromoacetone was done according to [13] with the following modification: the concentration of *Achromobacter* collagenase in the reaction mixture was 0.5 mg/ml. The reaction was carried out at 20°C, at pH 6.5, in 0.3 M Tris–HCl buffer which was 0.02 M in $CaCl_2$ and 1 M in NaCl, constant pH was maintained by addition of 3 N NaOH. After 3 h incubation the samples were dialysed overnight and lyophilised.

2.4. *Inhibition by Zn^{2+}*

The inhibition of collagenase (1 mg/ml) by different molar concentrations of $ZnCl_2$ was performed in 0.45% dimethylformamide (pH 7.0). The samples were incubated for 2 h at 20°C under stirring and pH-control. The residual activity was determined by standard assay (section 2.2.) using substrate solutions containing the respective concentrations of Zn^{2+}.

2.5. *Inhibition by EDTA*

The inhibition was done as in [5], at pH 7.0, in the presence of 10^{-2} M EDTA. The enzyme (1 mg/ml) was incubated with the inhibitor for 2 h at 20°C prior to the treatment by bromoacetone.

3. Results and discussion

3.1. *Inactivation of the enzyme by bromoacetone*

The choice of conditions for the alkylation of collagenase was determined by the stability of the enzyme (pH 6–8.5) and by the specificity of the reagent. It was found [13] that, at pH 7.5, besides histidine an extensive modification of lysines occurs, whereas, at pH 6.5, the alkylation of histidine residues is specific. All our studies of collagenase alkylation with bromoacetone were therefore performed at pH 6.5. As shown in fig.1, 75% loss of activity takes place with a 100-fold reagent enzyme molar ratio. A higher molar excess of bromoacetone (up to 10 000-fold) increases the final inactivation to 92%. In the presence of three different concentrations of reagent the reaction approaches completion within 3 h at 20°C. Comparison of the amino acid composition of collagenase before and after 3 h treatment with bromoacetone shows that one histidine residue disappeared in the presence of 100 and 1000 molar excess of the reagent whereas three histidine residues are lost in the presence of 10 000 molar excess of reagent (table 1). No significant change in the content of other amino acids was observed. Therefore the modification of one histidine residue with bromoace-

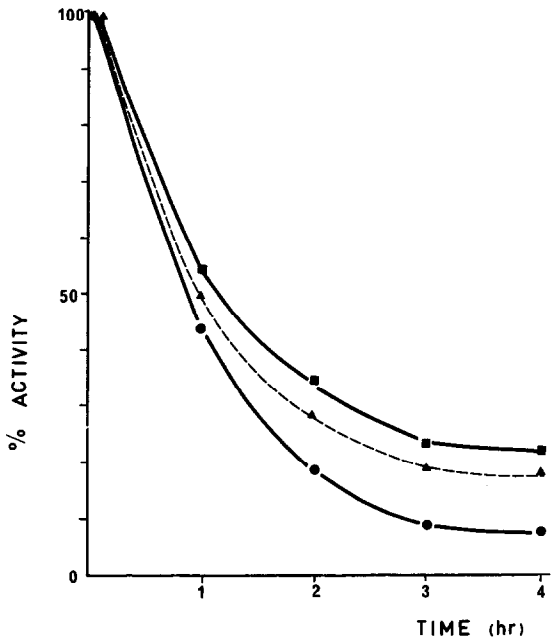

Fig.1. Effect of treatment with bromoacetone on the activity of *Achromobacter* collagenase. Experiments were carried out as in section 2.3. Enzyme was treated with 100 (■——■), 1000 (▲– –▲) and 10 000 (●——●) molar excess of bromoacetone.

Table 1
Amino acid analysis of native and modified *Achromobacter* collagenase

Amino acid	Native collagenase[a]	Bromoacetone/collagenase molar ratio			
		Modified collagenase		EDTA pretreated and modified collagenase[b]	
		100:1	1000:1	10 000:1	100:1
Half-cystine	7.7	n.d.	7.6	n.d.	n.d.
Aspartic acid	76.0	76.0	76.0	76.0	76.0
Threonine	50.0	49.3	48.8	49.8	48.0
Serine	54.0	53.4	52.8	54.3	54.0
Glutamic acid	87.0	88.7	90.0	89.0	87.4
Proline	13.9	13.7	14.0	13.5	14.2
Glycine	54.0	53.9	55.0	54.0	54.6
Alanine	52.0	52.3	51.0	51.3	52.1
Valine	37.0	37.2	36.6	36.0	37.0
Methionine	15.0	n.d.	14.5	15.3	n.d.
Isoleucine	31.0	31.5	32.0	31.8	32.4
Leucine	48.0	48.4	46.5	49.0	50.0
Tyrosine	34.8	35.0	34.0	34.6	32.0
Phenylalanine	30.0	29.8	30.0	29.6	30.0
Lysine	21.0	21.4	21.0	21.2	21.0
Histidine	17.0	15.9	15.8	13.9	9.0
Arginine	18.0	18.0	18.0	18.4	17.0
Tryptophan	16.3	n.d.	17.0	n.d.	n.d.

[a] Calculated for the mol. wt 70 000 according to [5]
[b] Calculated for the mol. wt 2 × 35 000 [5]

n.d., Not determined

tone results in 75% loss of collagenolytic activity and the modification of two additional histidines does not increase the inactivation substantially.

The evidence for the role of histidine in thermolysin and in the neutral proteinase from *Bacillus subtilis* was drawn from its selective modification with ethoxyformic anhydride [6]. In the case of *Achromobacter* collagenase, we preferred to undertake the modification with bromoacetone instead of ethoxyformic anhydride because the ethoxyformylated derivative is too unstable to be isolated and identified [14]; on the contrary alkylation with bromoacetone leads to the formation of a stable product [13] which could be later characterized by sequence analysis.

3.2. *Protection by prolyl–leucyl–sarcosyl–proline against reaction with bromoacetone*

The tetrapeptide Pro–Leu–Sar–Pro is a competitive inhibitor of *Achromobacter* collagenase [15]. When the standard assay using the pentapeptide 4-phenylazo-Cbz–Pro–Leu–Gly–Pro–D-Arg was effected in the presence of 1000 molar excess of the competitive inhibitor, the residual activity was found to be 42%. This residual activity was not changed after treatment of the inhibited enzyme for 3 h with 1000 molar excess of bromoacetone. After incubation the sample was dialysed against distilled water and lyophilised. Amino acid analyses of three independent experiments showed no detectable changes in histidine content in comparison with the non-treated collagenase. This protection by the peptide inhibitor against inactivation by bromoacetone indicates that the same histidyl residue is important for inhibitor binding and for the reaction with bromoacetone.

3.3. *Inhibition of collagenolytic activity by Zn^{2+}*

It was shown that in the bacterial Zn-metalloproteinase thermolysin two histidyl residues (142 and

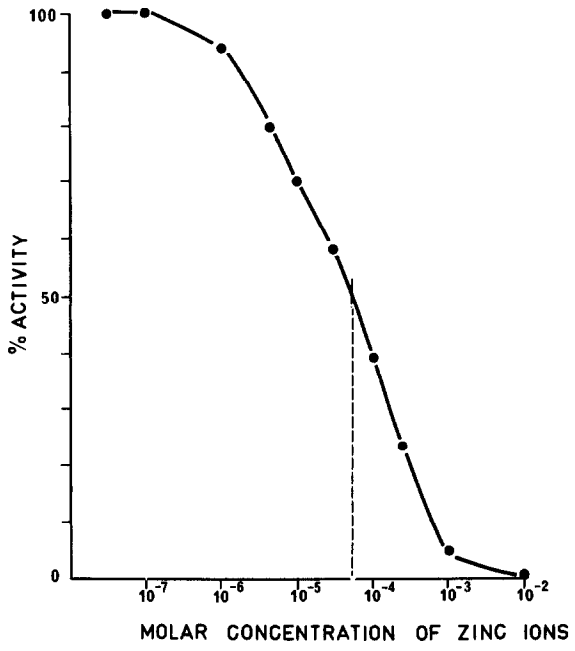

Fig.2. Inhibition of *Achromobacter* collagenase by Zn^{2+}.

146) serve as zinc ligands and a third (His-231) is free to participate directly in catalysis [16,17]. The same histidine 231 was shown to bind metals in the crystals [18].

The results of fig.2 show that *Achromobacter* collagenase is completely inhibited in the presence of 10^{-2} M $ZnCl_2$ and that 50% inhibition takes place at 7×10^{-5} M Zn^{2+}. Similar values were reported for 50% inhibition of thermolysin and neutral protease A from *Bacillus subtilis* [6].

When collagenase was treated by a 10 000 molar excess of bromoacetone in the presence of 10^{-4}–10^{-2} M $ZnCl_2$, the amino acid analysis of the inactivated enzyme showed the disappearance of 3 histidyl residues, a result which is in agreement with the data of table 1 and which shows that the added Zn^{2+} have no protective effect on the extent of the modification. Nevertheless it is difficult to conclude whether the modified histidines are the same as in the active enzyme. Further structural studies could bring an answer to this question.

3.4. *The molecular weight of modified collagenase*

As shown [5], native collagenase of mol. wt 70 000 lost its activity after dissociation into subunits of mol. wt 35 000. In order to prove that the decrease of activity by substitution could not be due to dissociation, the molecular weight values of the samples were checked by SDS-electrophoresis. The results showed that under all the experimental conditions listed above (section 3.1–3.3) the enzyme conserved its dimeric form.

3.5. *Alkylation of collagenase inhibited by EDTA*

Collagenase is inhibited by 0.1 M EDTA. Under these conditions the enzyme dissociates into 2 subunits [5]. This inactive dissociated apoenzyme was treated by 100 molar excess of bromoacetone in the same conditions as the active enzyme. The results in table 1 show that 8 histidine residues/70 000 mol. wt were modified. Only 1 histidine was modified under the same conditions in the active enzyme. This suggests that the dissociation of *Achromobacter* collagenase into subunits increases the accessibility of histidine residues and that the structure of inactive apoenzyme is not the same as that of the active form.

Acknowledgements

The authors are grateful to Miss O. Siffert for the peptide inhibitor sample, to Mr N.-T. Tong for the amino acid analyses and to Mr L. Bagilet for the gel electrophoreses. This study was supported by grant no. 77.7.0282 of DGRST and partly by the Fondation pour la Recherche Médicale Française.

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