Biochimica et Biophysica Acta, 615 (1980) 436—448

BBA 69114

CHEMICAL MODIFICATIONS OF *ACHROMOBACTER* COLLAGENASE AND THEIR INFLUENCE ON THE ENZYMIC ACTIVITY

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(Received April 1st, 1980)

Key words: Collagenase; Chemical modification; Active site; (Achromobacter)

Summary

A study of the influence of chemical modifications on the activity of *Achromobacter iophagus* collagenase (EC 3.4.24.8) has led to the following conclusions: a modification of 4 out of 80 COOH groups with carbodiimide led to 90% loss of enzymic activity. A 70% inactivation was found after modification of two tyrosines out of 30 with tetranitromethane. The modification of four to six tryptophans out of 16 with 2-hydroxy-5-nitrobenzyl bromide decreased enzyme activity to 36%. This inactivation is accelerated in the presence of collagen. An increase of reagent/enzyme molar ratio led to a modification of 16 tryptophan residues and denaturation of *Achromobacter* collagenase.

A modification of two arginines out of 18 with 1,2-cyclohexanedione and eight NH_2 groups out of 24 with 2,3-dimethyl maleic anhydride does not change the collagenolytic activity. All NH_2 groups become available for 2,3-dimethyl maleic anhydride after dissociation of the dimer. A possible analogy of hydrolytic site of collagenase with that of two other known bacterial metalloproteinases (thermolysin and *Bacillus subtilis* neutral proteinase (EC 3.4.24.4)) is discussed.

Introduction

The collagenase from *Achromobacter iophagus* is extracellular Zn-metalloproteinase (EC. 3.4.24.8) [1,2] whose synthesis is induced in the bacterial cul-

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Abbreviations: DHCH-arginine, N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; DNPED, *N*-(2,4-dinitrophenyl)ethylenediamine; DMMA, 2,3-dimethyl maleic anhydride; HNB-bromide, 2-hydroxy-5-nitrobenzyl bromide.

ture by collagen or its high molecular weight fragments [3]. The active enzyme is composed of two subunits of molecular weight 35 000. Each subunit consists of a single polypeptide chain. A unique N-terminal sequence indicates that the two subunits are identical at least in the N-terminal part of the chain. Only the dimeric form of the enzyme is active [4]. Our previous data have shown that histidine is involved in the active site of *Achromobacter* collagenase [5].

Two studies on the influence of the effects of specific chemical modifications on the activity of another bacterial Zn-metalloproteinase from *Clostridium histolyticum*, have been published previously. Photooxidation of 25% of histidines inactivated *Clostridium* collagenase, while Ca^{2+} had a protective effect [6]. Acylation of amino and hydroxyl groups by succinic anhydride or by acetylimidazol in the *Clostridium* collagenase resulted in total loss of enzymic activity although the presence of a peptidic substrate protected the enzyme from inactivation [7].

The aim of this work is to determine amino acid residues which are important for the enzymic function of *Achromobacter* collagenase as well as to compare this enzyme with the other known Zn-containing metalloproteinases.

Materials and Methods

Materials. Crude collagenase from *Achromobacter iophagus* of specific activity 0.3 μkat/mg was purchased from Institut Pasteur Production. Homogeneous *Achromobacter* collagenase of specific activity 2.0 μkat/mg was obtained by chromatography on DEAE-cellulose and Sephadex G-100 as described previously [2,6]. Insoluble collagen was purchased from Worthington Biochemical Corporation. Synthetic substrate for the collagenase assay, 4-phenyl-azobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine dihydrate (Pz-Pro-Leu-Gly-Pro-D-Arg) and 1,2-cyclohexanedione were purchased from Fluka. 2,3-dimethyl maleic anhydride was a product of Aldrich Chem. Co. 2-Hydroxy-5-nitrobenzyl-bromide, tetranitromethane and 1-ethyl-3(3-dimethyl aminopropyl)carbodiimide hydrochloride were obtained from Sigma. *N*-(2,4-dinitrophenyl)ethylenediamine HCl was a product of Calbiochem.

Enzyme assay and analytical methods. Collagenase activity was measured colorimetrically using Pz-Pro-Leu-Gly-Pro-D-Arg, according to Wünsch and Heidrich [9]. Numerical data have been recalculated on the basis of 1 nkat = 90 units.

Protein concentrations were determined by the method of Lowry et al. [10].

SDS-polyacrylamide gel electrophoresis was performed according to Ames [11] in 25 mM Tris/0.2 M glycine buffer (pH 8.3) which was 0.1% in SDS. The proteins were heated for 5 min at 100°C in 1% β-mercaptoethanol containing 3% SDS, prior to electrophoresis. Phosphorylase *b* (97 000), bovine serum albumin (68 000), aldolase (subunit 40 000), chymotrypsinogen (25 700) and parvalbumin (12 000) were used as standard markers.

Polyacrylamide gel electrophoresis at pH 8.3 was done according to Uriel [12].

Amino acid analyses. Amino acid analyses were performed with a Beckman Multichrome amino acid analyser. Protein hydrolyses were carried out at 110°C under nitrogen. The cysteine and methionine contents were obtained after

oxidation with performic acid [13]. Tryptophan was determined after hydrolysis by 4 N methanesulphonic acid, according to Liu and Chang [14].

The content of tyrosines and 3-nitrotyrosines was determined according to Imhoff and Keil [15] after hydrolysis for 15 h in the presence of 0.2% phenol. A peak of 3-nitrotyrosine appeared after phenylalanine [16].

Arginine and DHCH-arginine content of the collagenase modified with cyclohexanedione was determined according to Patty and Smith [17]. Acid hydrolyses were performed at 110°C in 6 N HCl for 24 h in the presence of mercaptoacetic acid.

NH_2 groups were evaluated by the trinitrobenzene sulfonic acid method [18].

Modification of carboxyl groups with EDC and DNPED. Modification of carboxyl groups was done according to Hoare and Koshland [19]. Solid carbodiimide was added to a $1.25 \cdot 10^{-5}$ M collagenase solution in water. The pH 6.0 was maintained in the reaction mixture by addition of 0.01 N HCl.

When the reaction with carbodiimide was done in the presence of a coloured nucleophile DNPED, solid carbodiimide was added to a $1.25 \cdot 10^{-5}$ M solution of collagenase which already contained 3 mM DNPED. A solution of DNPED in water was prepared by dilution of 2% (v/v) solution of DNPED in dimethylformamide. The concentration of DNPED was determined using coefficient $\epsilon M_{360} = 15\,000$ for dinotrophenyl groups, according to Huc et al. [20]. The reaction was stopped by addition of 2 N acetic acid and the pH was adjusted to 6.0 [19]. The reaction mixture was then applied to a Sephadex G-25 column (20 × 2 cm) equilibrated with 0.01 M CH_3COOH which was made 1 M in NaCl, pH 6.0. The absorbance of the eluted fractions was measured at 280 and 360 nm. After enzyme assays, the fractions containing collagenase were pooled and dialyzed against H_2O at 4°C. The extent of modification was calculated as the ratio between the number of incorporated DNPED groups and protein concentration.

Modification of NH_2 groups with DMMA. This modification was performed according to Puigserver and Desnuelle [21] under the following conditions. *Achromobacter* collagenase in concentration 2.5 mg/ml was incubated at 0°C in 50 mM borate buffer which was made 200 mM in NaCl, at pH 9.0. Solid reagent was added with stirring, pH 9.0 was maintained by addition of 3 N NaOH.

The number of modified NH_2 groups were calculated from the difference between the number of free NH_2 groups before and after modification as evaluated by the trinitrobenzene sulfonic acid method [18].

Deacylation of DMMA-collagenase was performed by incubation of the modified protein at pH 6.0, in 0.1 M dipotassium phosphate for 6 h, at 0°C.

Modification of arginine residues. Modification of arginine residues was performed according to Patty and Smith [22]. Collagenase (final concentration $1.25 \cdot 10^{-5}$ M) was treated with 8—40 mM cyclohexanedione in 0.2 M sodium borate at pH 9.0 and 37°C, in the dark under nitrogen. Aliquots were removed at intervals, diluted into cold 5% acetic acid and subsequently dialyzed against 5% and 1% acetic acid at 4°C. After lyophilisation the samples were submitted to SDS-polyacrylamide gel electrophoresis and amino acid analysis. Enzyme assays were done before treatment with acetic acid, because at pH below 4.5

Achromobacter collagenase is irreversibly inactivated.

Reaction of collagenase with HNB-bromide. This modification was done according to Horton and Koshland [23]. In a typical experiment the concentration of collagenase was $1.25 \cdot 10^{-5}$ M. The enzyme was solubilized in 0.1 M Tris-HCl, pH 7.0 which was 20 mM in $CaCl_2$ and 1 M in NaCl. Reagent/enzyme molar ratios used varied from $15 \cdot 10^3$ to $15 \cdot 10^4$. The reagent was dissolved in 0.4 ml dry acetone and added with rapid stirring within 5 min at 22°C. The pH was maintained at 7 ± 0.01 by the addition of 1 N NaOH. After 20 min, the insoluble product of hydrolysis of excess reagent was removed by centrifugation.

In the experiment carried out in the presence of collagen, *Achromobacter* collagenase was incubated with 10-fold molar excess of collagen at 22°C for 10 min, prior to the modification.

Reaction of collagenase with tetranitromethane. The collagenase was modified with tetranitromethane according to Shljapnikov et al. [24]. The enzyme (0.5 mg/ml) was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, which was 20 mM in $CaCl_2$. Tetranitromethane was dissolved in 95% ethanol prior to being added to the enzyme solution. The pH was maintained at 8.0 by addition of 1 N NaOH. Aliquots were removed at intervals for enzyme assays. The reaction was stopped by chilling to 0°C. The reaction mixture was applied to a Sephadex G-25 column (20 × 2 cm) equilibrated with Tris-HCl 0.1 M, which was 20 mM in $CaCl_2$ and 1 M in NaCl, pH 8.0. The flow rate of elution was 24 ml/h and fraction vol. 2 ml. The absorbance of eluate was measured at 280 and 428 nm. The fractions containing collagenase activity were pooled together and dialyzed against 2.5 mM Tris-HCl, pH 8.0. The protein concentration was determined according to Lowry et al. [10] using bovine serum albumin as a standard solution. The aliquots were removed for SDS-polyacrylamide gel electrophoresis and amino acid analyses.

Results

Modification of charged amino acid residues

Modification of carboxyl groups with EDC. The results of Fig. 1 show that in the presence of $8 \cdot 10^{-2}$ M EDC (EDC/enzyme molar ratio $6.4 \cdot 10^3$) inactivation of enzyme is rapid. After 20 min of incubation the residual collagenolytic activity was less than 1%. The increase of EDC concentration up to $20 \cdot 10^{-2}$ M does not substantially change the first part of the curve of inactivation, but residual enzymic activity less than 1% was found already after 10 min.

It is known that carbodiimide aside of its main reactivity towards carboxylic groups could also react with hydroxyls [25]. This reaction results in the formation of *O*-acylisourea derivatives of tyrosines which can be decomposed by treatment with hydroxylamine.

The collagenase inactivated to 97% by EDC was therefore dialyzed against 0.75 M NH_2OH at pH 7.0, and 4°C. The samples for the collagenolytic assay were removed after 2 h and 30 h. In both samples we did not find a reappearance of collagenolytic activity. Therefore, the 97—100% loss of enzymic activity after treatment with EDC can be attributed to the specific modification of carboxyl groups.

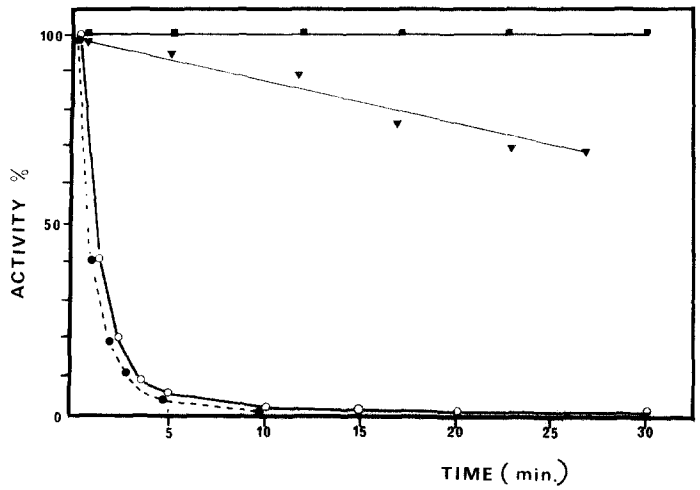


Fig. 1. Change in collagenase activity as a function of time of reaction with different concentrations of EDC. Enzyme conc. $1.25 \cdot 10^{-5}$ M; EDC concentration ■———■, none; ▼———▼, 3 mM; ○———○, 80 mM; ●- - - - -●, 200 mM.

Modification of carboxyl groups with carbodiimide in the presence of DNPED. The reaction which enables colorimetric identification of the number of modified carboxylic groups is the coupling of carboxyls with a coloured nucleophilic reagent *N*-2,4-(dinitrophenyl)ethylenediamine in the presence of carbodiimide [26].

The results of Fig. 2 give a comparison of collagenase inactivation in the

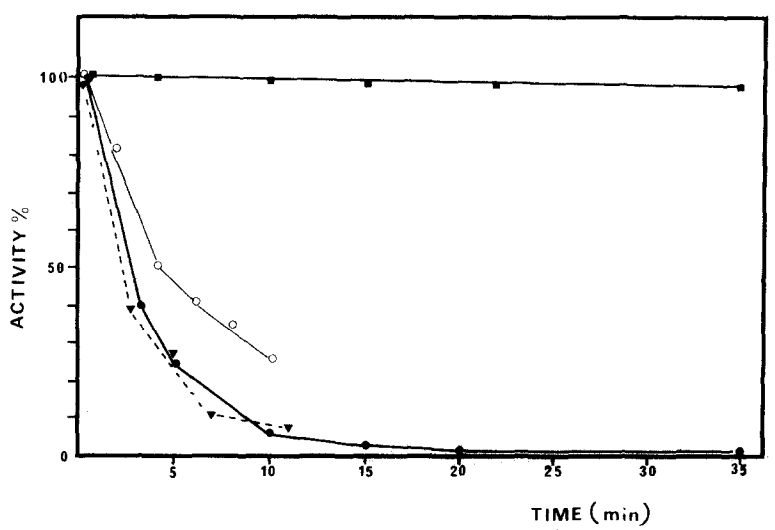


Fig. 2. Change in activity of *Achromobacter* collagenase during reaction with EDC in the presence of DNPED. The reaction conditions are as in Fig. 1. The collagenase sample was incubated with 9 mM DNPED in the absence of EDC (■———■); concentration of EDC was 50 mM, DNPED was 3 mM (○———○); EDC was 100 mM, DNPED was 3 mM (▼- - - - -▼); EDC was 100 mM and DNPED 9 mM (●———●).

presence of different concentrations of EDC and DNPED. It is evident that in this concentration region the enzyme inactivation depends on the concentration of EDC but not on that of DNPED.

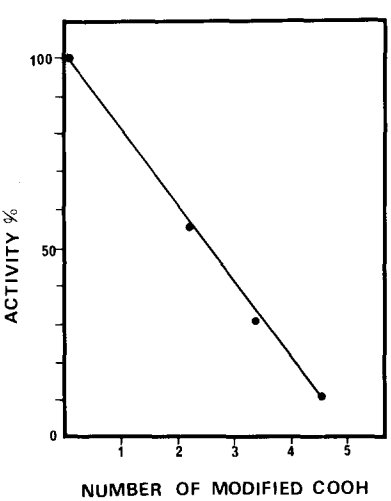
The sample of *Achromobacter* collagenase which was incubated under the same conditions in $9 \cdot 10^{-3}$ M DNPED but in the absence of carbodiimide did not show any loss of enzymic activity. Therefore, nucleophilic diamine alone does not influence the collagenolytic activity, and the inactivation of enzyme is proportional to the carbodiimide concentration.

A colorimetric determination of the number of the carboxyl groups modified in the presence of EDC and DNPED is summarized in Fig. 3. It is evident that modification of four to five carboxyl groups for the collagenase dimer (M_r 70 000) is followed by 90% loss of collagenolytic activity. The results of SDS-polyacrylamide gel electrophoresis have shown that under the conditions of the modification the enzyme remains in its dimeric form.

The electrophoretic mobility of the samples with a different number of modified carboxylic groups shows (Fig. 4) that the migration of the samples is proportional to the number of the modified carboxyls and that modified samples were homogeneous as concernes the number of modified residues.

Modification of lysine residues with DMMA. The *Achromobacter* collagenase of molecular weight 70 000 contains 22 lysine residues in the active dimer and two N-terminal NH_2 groups (one for each subunit).

The results of Table I show that 10 out of 24 NH_2 groups are modified after 1 h of incubation at pH 9.0 with 80 molar excess of DMMA (five groups for each subunit). Even after 4 h of incubation in the presence of 320 molar



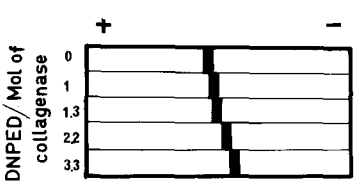


Fig. 3. Dependence of collagenase inactivation upon the number of carboxyl groups modified with carbodiimide in the presence of DNPED. EDC/collagenase molar ratio was 160 for 56% residual activity, 800 for 32% residual activity and $8 \cdot 10^3$ for 10% residual activity. DNPED/collagenase molar ratio was 240 in all samples. The incubation was done at pH 6.0 for 10 min. The specific activity and number of DNP groups incorporated was measured after desalting of each sample on Sephadex G-25 column.

Fig. 4. Change in the electrophoretic mobility of *Achromobacter* collagenase after modification of different number of COOH groups. The electrophoresis was performed at pH 8.3.

TABLE I

MODIFICATION OF NH_2 GROUPS DURING REACTION OF COLLAGENASE WITH DIMETHYL MALEIC ANHDYRIDE

Number of free NH_2 groups after reaction were evaluated by the trinitrobenzene sulfonic acid method [16]. Number of NH_2 groups modified were determined as the difference between total number of NH_2 groups and number of free NH_2 groups. Molecular weight was determined by SDS-polyacrylamide gel electrophoresis.

Time (h)	DMMA/Collagenase molar ratio	Specific activity (%)	Number of free NH_2 groups after reaction	Number of NH_2 groups modified	M_r of collagenase
0	0	100	24	0	70 000
1	80	97	14	10	70 000
4	80	92	14.1	10	70 000 + traces 35 000
1	320	95	13.7	10	70 000
4	320	92	14.2	10	70 000 + traces 35 000
4	0	91	24	0	70 000 + traces 35 000

excess of reagent no further change in content of NH_2 groups was observed.

The incubation of collagenase with or without DMMA under these conditions is followed by a decrease of only 3—8% of the enzymic activity. The results of SDS-polyacrylamide gel electrophoresis have shown that the molecular weight of both samples was not substantially changed under reaction conditions. Therefore, modification of 10 NH_2 groups in the collagenase dimer does not influence its enzymic activity and this modification is not followed by dissociation into subunits.

Incubation of collagenase at pH 9 for 20 h results in the dissociation of about 50% of collagenase dimer into subunits and in a 45% decrease of collagenolytic activity (Table II).

This partially dissociated sample was incubated with 80 molar excess of DMMA for 1 h and then applied to the Sephadex G-100 column. As shown in Fig. 5, three major peaks were obtained.

TABLE II

MODIFICATION OF NH_2 GROUPS IN PARTIALLY DISSOCIATED COLLAGENASE

Molecular weight as determined by SDS-polyacrylamide gel electrophoresis. Number of free NH_2 groups as determined using tetranitrobenzene sulfonic acid. n.d., not determined.

Sample	M_r	Number of free NH_2 groups	Number of NH_2 groups modified	Residual activity (%)	Yield (%)
Collagenase (non-treated)	70 000	24 (22 Lys + 2 NH_2)	0	100	—
Reaction mixture *	70 000 + 35 000	8	16	45	≃50 ≃50
Sephadex G-100 *					
peak I	dead vol.	n.d.	n.d.	0	9.8
peak II	70 000	16	8	100	42.9
peak III	35 000	0	24	0	47.3

* As described under Fig. 5.

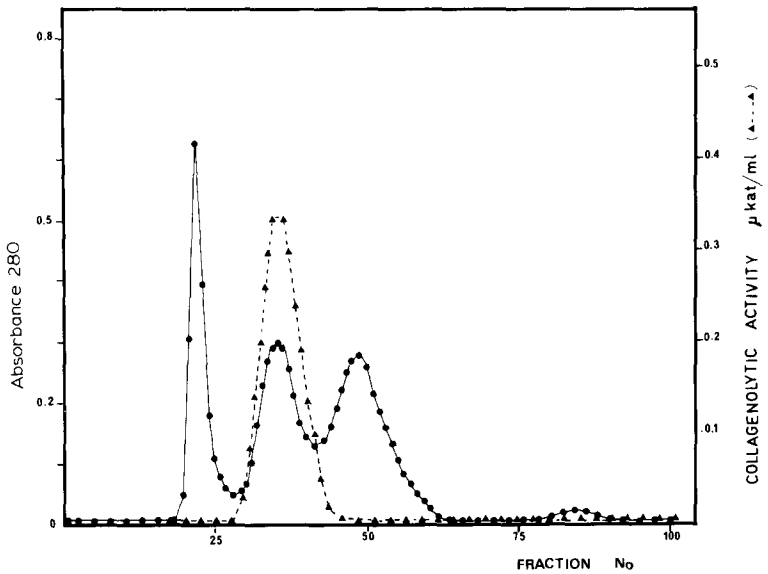


Fig. 5. Gel filtration of *Achromobacter* collagenase partially dissociated at pH 9.0 after its reaction with DMMA. 15 mg *Achromobacter* collagenase were incubated in 6 ml of 50 mM borate buffer, pH 9.0, which was 200 mM in NaCl for 20 h. Then, DMMA was added. Collagenase was incubated with 80 molar excess of DMMA for 1 h and then applied to the Sephadex G-100 column (69 × 2.2 cm) which was equilibrated and eluted with 50 mM borate buffer, pH 9.0, containing 1 M NaCl and 0.1 M histidine. The flow rate was 26 ml/h, 3-ml fractions were collected.

Peak I (9.8% of total protein applied to the column) was inactive. The results of SDS-polyacrylamide gel electrophoresis have shown the presence of products of different molecular weights. Therefore, we can assume that it contains the product of nonspecific association of differently degraded fragments.

Peak II (42.9%) contained all collagenolytic activity of the sample applied to Sephadex G-100 column, whereas peak III (47.3%) was inactive. The results of SDS-polyacrylamide gel electrophoresis have confirmed the values of molecular weight 70 000 for peak II and 35 000 for peak III. The amino acid composition of proteins from both peaks were identical.

The determination of NH_2 groups by reaction with 2,4,6-trinitrobenzene sulfonic acid showed the presence in peak II of 16 free NH_2 groups (Table II). Therefore, eight out of 24 NH_2 groups were modified in the dimer (4 per monomer). On the other hand, in the peak III, all NH_2 groups were modified. Therefore, eight out of 12 NH_2 groups of each subunit become available for modification with DMMA only after dissociation of the dimer.

Incubation of the DMMA-modified subunit (peak III) under conditions of deacylation led neither to appearance of collagenolytic activity nor to reconstitution of dimer, as shown by SDS-polyacrylamide gel electrophoresis.

Modification of arginine residues with 1,2-cyclohexanedione. Incubation of the collagenase at 37°C, at pH 9.0, for 1 h with either 12- or 30-fold molar excess of reagent resulted in the modification of two arginine residues out of 18 present in the dimer (70 000). A 10% loss of enzymatic activity was observed in both samples which were incubated at pH 9.0, in the presence or

absence of reagent. This small decrease of the activity is conditioned by the alkaline pH of the reaction. (The same effect was observed during modification of lysines).

Therefore, a modification of two arginine residues in the dimer of collagenase does not influence its enzymic activity.

Modification of hydrophobic amino acid residues

Modification of tryptophan residues with HNB-bromide. The results of Table III show that after modification of four to six tryptophan residues out of 16 the collagenase is still soluble but its specific activity is decreased to 35.7%. Modification of 16 tryptophan residues obtained with a higher molar excess of the reagent (1320-fold) is followed by denaturation and precipitation of the enzyme. A presence of collagen increases the inactivation of *Achromobacter* collagenase by HNB-bromide (Fig. 6).

Modification of tyrosine residues with tetranitromethane. The degree of inactivation of *Achromobacter* collagenase during its incubation with tetranitromethane is dependent on the reagent concentration (Fig. 7). In the presence of $3.2 \cdot 10^3$-fold molar excess of tetranitromethane for 3 min, a 70% loss of enzymic activity is accompanied by a modification of two tyrosine residues out of 30 present in the dimer molecule. This value was obtained spectrophotometrically and by determination of nitrotyrosines in the amino acid analyser. Incubation for 30 min under the same conditions led to the modification of 12 tyrosine residues and to an almost complete inactivation (the residual activity 0.2%). As shown in Fig. 7 the presence of competitive inhibitor, 0.1 M histidine, does not change the rate of the inactivation by tetranitromethane.

On the other hand, incubation of the collagenase for 40 min in the presence of 0.1 M histidine but in the absence of tetranitromethane does not change the enzymic activity. In order to demonstrate that inactivation of *Achromobacter* collagenase after incubation with tetranitromethane can be attributed to the specific modification of tyrosines, we have verified the integrity of histidines and tryptophans after reaction. The results of amino acid analysis have shown no changes in the content of those residues. The analysis of SDS-polyacrylamide gel electrophoresis has also shown that nitration of the enzyme does not change its molecular weight: no dissociation into subunits was observed even after 90 min incubation with $3.2 \cdot 10^3$-fold molar excess of tetranitromethane.

Therefore, a modification of two tyrosine residues per molecule of *Achro-*

TABLE III

INFLUENCE OF TRYPTOPHAN MODIFICATION ON THE COLLAGENASE ACTIVITY

Number of Trp groups modified were calculated from tryptophan content before and after reaction (average from three analyses). HNB-Br, HNB-bromide.

Collagenase concentration	HNB-Br/Trp molar ratio	Residual activity (%)	Number of Trp groups modified	Collagenase solubility
$1.25 \cdot 10^{-5}$ M	380	35.7	4—6	soluble
$1.25 \cdot 10^{-5}$ M	1320	4	16	denatured

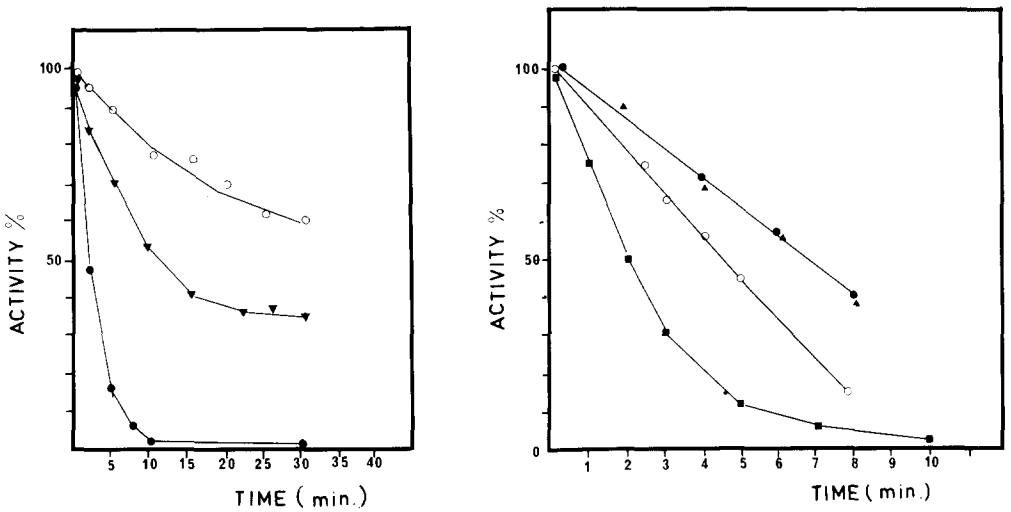


Fig. 6. Kinetics of collagenase inactivation during reaction with HNB-bromide. Collagenase $1.25 \cdot 10^{-5}$ M (solution in 0.1 M Tris-HCl/20 mM $CaCl_2$/1 mM NaCl, pH 7) was incubated with: $3 \cdot 10^{-2}$ M HNB-bromide (○———○); $12 \cdot 10^{-2}$ M HNB-bromide (▼———▼); collagen 10^{-4} M, then HNB-bromide was added to its final concentration $12 \cdot 10^{-2}$ M (●———●).

Fig. 7. Kinetics of collagenase inactivation in the presence of tetranitromethane. Collagenase $6 \cdot 10^{-6}$ M was incubated in 0.1 M Tris-HCl/20 mM $CaCl_2$/1 M NaCl, pH 8.0 in different concentrations of tetranitromethane. Tetranitromethane 1.1 mM (●———●); (b) 1.1 mM in the presence of 0.1 mM histidine (▲———▲); 8 mM tetranitromethane (○———○); 25 mM tetranitromethane (■———■).

mobacter collagenase is followed by rapid loss of enzymic activity: 70% of initial enzyme activity was lost in 3 min.

Discussion

Amongst the metallopeptidases the most thoroughly characterized are the bacterial endopeptidase thermolysis and carboxypeptidase A from beef liver. The primary and three-dimensional structures of both enzymes are well known [27—30]. This, together with the data on the chemical modifications, allowed us to make a conclusion on the identity of two histidines and one glutamic acid residue which fixed the ligand, one ion of Zn^{2+}.

In the case of another bacterial Zn-metalloproteinase, neutral proteinase from *B. subtilis*, a study of its primary structure [31] as well as of the influence of chemical modifications on its activity, make plausible the hypothesis that neutral proteinase and thermolysin possess large similarities in their three-dimensional structures as well as in their catalytic site [32].

Therefore, the similarities of the molecular weights, metal-dependence and amino acid composition on one hand (Table IV) and the similarity in the amino acid residues, indispensable for the enzymic activity on the other hand (Table V), justify the hypothesis that organisation and function of the hydrolytic site of *Achromobacter* collagenase could be similar to those of thermolysin and neutral proteinase.

Nevertheless, the difference between *Achromobacter* collagenase and the

TABLE IV

A COMPARISON OF AMINO ACID COMPOSITION, MOLECULAR WEIGHTS AND METAL DEPENDENCE OF BACTERIAL METALLOPROTEINASES:THERMOLYSIN, *ACHROMOBACTER* COLLAGENASE AND NEUTRAL PROTEINASE FROM *BACILLUS SUBTILIS*

Amino acid	Thermolysin [27]	Collagenase subunit [4]	Neutral proteinase [33]
Lys	11	11	17
His	8	8	5
Arg	10	9	8
Asp + Asn	44	38	48
Thr	25	24	29
Ser	26	27	31
Glu + Gln	21	43	27
Pro	8	7	9
Gly	36	27	30
Ala	28	26	28
Half-Cys	0	4	0
Val	22	18	19
Met	2	3	4
Ile	18	14	13
Leu	16	25	21
Tyr	28	15	22
Phe	10	13	11
Trp	3	8	3
M_r	34 000	35 000	35 000
Metal dependence	Zn + Ca	Zn + Ca	Zn + Ca

two other bacterial metalloproteinases is that collagenase is active in its dimeric form only. This raises the question whether there are on or two active sites in *Achromobacter* collagenase. In our previous work one Zn^{2+} ion was found in the dimer [2]. The results of this work obtained with the chemical modifications of tyrosines and COOH groups, as well as our previous results obtained with the modification of histidines (Table V), support the hypothesis that only one active site is present in the dimer. We could not yet answer the question as to whether the hydrolytic site is common for two subunits or whether it is situated in one subunit which takes an active conformation after binding of another one.

The results obtained with dimethylmaleylation indicate that eight lysines in each subunit of 35 000 become available for the reagent after dissociation of collagenase dimer. Therefore, lysines are either directly involved in the interaction between subunits or they are sterically protected in the dimer.

Rapid inactivation of the collagenase in the presence of collagen during the tryptophan modification suggests that conformational changes take place in enzyme-substrate complex.

This work presents the first information about possible structural relationships between the hydrolytic site of *Achromobacter* collagenase and two other known bacterial metalloproteinases, thermolysin and *B. subtilis* neutral proteinase. But the confirmation of this hypothesis can be achieved only after more profound structural work.

TABLE V

INFLUENCE OF CHEMICAL MODIFICATIONS ON THE ACTIVITY OF *ACHROMOBACTER* COLLAGENASE

Amino acid	Residues per 70 000	Reagent	Molar excess	Residues modified per 70 000	Residual activity (%)	Thermolysin(T) and *B. subtilis* proteinase A(S) *
Arg	18	1,2-cyclohexanedione	12	2	100	Arg_{203} (T,S)
			30	2	100	
Lys + NH_2 term.	22 + 2	Dimethyl maleic anhydride	80	8	100	
COOH	80	Carbodiimide + DNP-ethylenediamine	$8 \cdot 10^3$	4	10	Glu_{143}, Glu_{166}, Asp_{170} (T,S); Asp_{226} (T)
Trp	16	2-OH-5-NO_2-benzyl bromide	$3.8 \cdot 10^2$	4–6	36	
			$1.3 \cdot 10^3$	16	insoluble	
Tyr	30	Tetranitromethane	$3.1 \cdot 10^3$ (3 min)	2	30	Tyr_{157} (T,S)
			$3.1 \cdot 10^3$ (30 min)	12	0.2	
His [5]	16	Bromoacetone	10^2	1	25	His_{142}, His_{146}, His_{231} (T,S)
			10^3	1	25	
			10^4	3	8	
Cys [4]	8	Dithiothreitol + vinylpyridine	10	8	0	

* Residues located in the active site or Zn-binding site [31,32].

Acknowledgements

The authors thank Mr. Bagilet for polyacrylamide gel analyses and Mr. Tong for the amino acid analyses. This work was sponsored in part by the grants No. 72.7.0742 and 76.7.0079 from D.G.R.S.T.

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