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SUBUNIT STRUCTURE OF *ACHROMOBACTER* COLLAGENASE

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Summary

The highly active form of collagenase (EC 3.4.24.3) from *Achromobacter iophagus* (specific activity 2 μkat/mg) has a molecular weight of 70 000 and the sedimentation coefficient $s_{20,w}$ = 4.4 S. It is composed of two subunits of molecular weight 35 000 and $s_{20,w}$ of 2.9 S. The dissociation of the dimer under different conditions resulted in the complete and irreversible loss of enzymic activity. A unique N-terminal sequence Thr-Ala-Ala-Asp-Leu-Glu-Ala-Leu-Val- indicates that the two subunits are identical, at least in the N-terminal part of the polypeptide chain. Reduction and pyridylethylation of the subunit change neither molecular weight nor amino acid composition: therefore each subunit of molecular weight 35 000 consists of a single polypeptide chain.

Another active and homogeneous form of *Achromobacter* collagenase (specific activity 1.64 μkat/mg) gives a value for the apparent molecular weight of 80 000 on sodium dodecyl sulphate-polyacrylamide electrophoresis. It is also a dimer in which each of the two subunits of molecular weight 35 000 binds non-covalently a peptide of molecular weight 5000. The dissociation of this form of collagenase is also accompanied by irreversible loss of enzymic activity. The amino acid composition of the subunits which were isolated from both 70 000 and 80 000 collagenases is the same. The role of dimer-monomer equilibrium in the biological function of collagenase is discussed.

Introduction

Although the list of collagenases comprises at the present time dozens of representatives from widely different sources, it is difficult to discover any common structural pattern inside of this group of enzymes or in relation to other groups of Zn-metallo proteases. The data on their primary sequences have not yet been reported and their molecular weights fall within a wide range between 20 000 and 165 000. The high molecular weights of many collagenases

are thus a striking exception in the molecular weights found for most other Zn-metallo proteases.

For the bacterial collagenases from *Clostridium histolyticum* molecular weight values of 105—112 000 were found for collagenase A [1,2] and 50—57 000 for collagenase B. It was proposed that collagenase A is a dimer of collagenase B [2].

Lwebuga-Mukase et al. [3] have recently reported values of approximately 81 000 and 72 000 for the different collagenases isolated from the culture medium of *C. histolyticum*. Six forms of the same enzyme of molecular weight 100 000 were described by Kono [4].

However, there is no direct evidence to indicate whether the micro-organism produces isoenzymes, or that collagenase A and B are interconvertible in vitro or that one or both of the two forms are degraded during purification; the existence of *Clostridium* collagenase of much higher specific activity [5] than those used in the studies mentioned suggests the necessity of some additional work in this respect.

The first estimation of the molecular weight of another bacterial collagenase (EC 3.4.24.3), from *Achromobacter iophagus*, gave a value of 107 000—111 700 [6]. The purification of different active forms of this Zn-metallo enzyme to homogeneity in our laboratory [7,8] opened the way to a reinvestigation of the question of its molecular weight and to a study of its quaternary structure.

In a previous study [9] we described the induction of *Achromobacter* collagenase biosynthesis by macromolecular substrates and how the components of the culture medium influence quantitatively and qualitatively the composition of the crude collagenase.

In this communication we report that the enzyme is a dimer of molecular weight 70 000 which can be dissociated into two inactive subunits of molecular weight 35 000. Another homogeneous form of *Achromobacter* collagenase has a molecular weight of 80 000. It is also a dimer in which each of the two subunits of molecular weight 35 000 binds non-covalently a peptide of molecular weight 5000.

Materials and Methods

Materials

Batches of crude collagenase from *A. iophagus* of specific activity 0.30 μkat · mg^{-1} and 0.17 μkat · mg^{-1} (batch A and batch B) were obtained from Institut Pasteur Production. The preparations A and B of crude collagenase were isolated from the culture media as described previously [6]. In the case of batch A the culture medium contained 1.5% of commercial Atomised gelatine "P" AT 100 (Rousselot) which is a partial enzymatic hydrolysate of calf skin collagen containing the fragments of average molecular weight 5000—6000. In the case of batch B the culture was grown in the presence of 5% peptic hydrolysate of denatured collagen as described earlier [9]. Homogeneous *Achromobacter* collagenase of molecular weight 70 000 and specific activity 2.0 μkat · mg^{-1} was obtained from batch A by chromatography on DE-32 cellulose and Sephadex G-100 as described earlier [7,8]. Homogeneous collagenase of molecular

weight approx. 80 000 and specific activity 1.64 μkat · mg^{-1} was obtained from batch B by the same procedure.

4-Vinylpyridine was purchased from Sigma and redistilled before use. Other chemicals were of reagent grade or the highest purity available.

Collagenase assay

Enzyme activity was measured colorimetrically using 4-phenylazo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine dihydrate (Pz-Pro-Leu-Gly-Pro-D-Arg, Fluka) according to Wünsch and Heidrich [10]. Numerical data have been recalculated on the basis of 1 μkat = $9 \cdot 10^4$ units.

Molecular weight determinations

Sodium dodecyl sulphate (SDS) electrophoresis was performed using a 5—15% gradient in polyacrylamide gels according to Ames [11] in 25 mM Tris/0.2 M glycine buffer (pH 8.3) which was 0.1% with respect to sodium dodecyl sulphate. The proteins were heated for 5 min at 100° C in 1% β-mercaptoethanol containing 3% sodium dodecyl sulphate prior to electrophoresis.

The values of apparent molecular weights of collagenase and its subunits were obtained from semi-log plots of molecular weights of protein standards vs. their migration distance. As protein standards were used: phosphorylase B (93 000), aspartokinase I-homoserine dehydrogenase I from *Escherichia coli* K 12 (86 000), bovine serum albumin (68 000), aldolase (subunit 40 000), carbonic anhydrase (29 000) and trypsinogen (25 000).

The molecular weight determination of the enzyme and its subunits by analytical Sephadex G-100 chromatography was made using a column (60 × 2.2 cm) equilibrated with 0.3 M Tris · HCl buffer, pH 7, which was 2 mM in $CaCl_2$ and 1 M in NaCl; sample volume was 5 ml. As protein standards were used: phosphorylase B, bovine serum albumin, ovalbumin and trypsinogen.

The apparent molecular weight of the peptide associated with subunits in the collagenase of molecular weight 80 000 was determined by analytical Bio-Gel P-10 chromatography. The column (68 × 1.1 cm) was equilibrated with 6 M guanidine HCl. Insulin (molecular weight 6000) and insulin B chain (molecular weight 3800) were used as standards.

Sedimentation coefficients were determined in a Centriscan 75 (Measuring and Scientific Equipment, Crawley, U.K.) analytical ultracentrifuge. The experiments were done at 20° C, 50 000 ± 50 rev./min, at a protein concentration of 0.263 mg/ml in 0.3 M Tris · HCl, 20 mM $CaCl_2$ buffer at pH 7.5. Measurements were made at 280 nm, the values of $S_{20,w}$ were calculated from the observed sedimentation coefficient using the value of $\bar{v}$ = 0.719 ml/g.

Amino acid analyses

Amino acid analyses were performed with a Beckman Multichrome amino acid analyser. Protein hydrolysis was carried out at 110° C under nitrogen in sealed pyrex tubes. The cysteine and methionine content was obtained after oxidation with performic acid [12]. Tryptophan was determined after hydrolysis by methane-sulphonic acid, according to Liu and Chang [13]. *S*-Pyridylethylcysteine was determined using a commercial sample (Pierce Chemical Co.) as a standard.

S-pyridylethylation of the collagenase subunit

The protein was reduced and substituted with vinylpyridine according to Hermodson et al. [14]. After dialysis against 1% acetic acid the *S*-pyridylethyl protein was lyophilized.

Sequence analysis

Automated Edman degradation was performed in a Beckman 890 C sequencer using dimethylbenzylamine buffer [15]. The phenylhydantoins were determined by gas chromatography [15], high pressure chromatography [16] and thin layer chromatography [17].

Results

A. Achromobacter collagenase of M_r 70 000

Molecular weight. The crude collagenase from batch A has a specific activity of 0.3 μkat · mg^{-1}. After purification of the enzyme to homogeneity, its specific activity was increased to 2 μkat · mg^{-1}. Filtration of this collagenase on a Sephadex G-100 column gives a single peak corresponding to an apparent molecular weight of 70 000. When the same enzyme sample was submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate after treatment with mercaptoethanol, a single band corresponding to an apparent molecular weight of 70 000 appeared. The same preparation gave, in the ultracentrifuge, the sedimentation coefficient $s_{20,w} = 4.4$.

Dissociation at alkaline pH. The collagenase of molecular weight 70 000 was incubated at 4°C at pH 10.5 in 0.1 M glycine buffer containing 0.28 M LiCl. Enzymatic activity disappeared within the first 5 min of the incubation. After 2 h a sample of the incubation mixture showed the collagenase band and a new band of apparent molecular weight of 35 000 (Fig. 1) in SDS electrophoresis. The conversion of the collagenase into this product of half the original molecular weight was complete within 16 h of incubation. The rapid loss of enzymatic activity and the slow dissociation of the dimer to the monomer are, therefore, independent processes which exclude the possibility that the observed effect is an autolytic degradation.

The subunit of molecular weight 35 000 was purified from the traces of non-dissociated dimer by gel filtration on Sephadex G-100 in the same buffer. The solution was concentrated and desalted in an Amicon apparatus with the membrane UM-10 and lyophilized. Its amino acid composition is the same as that of the original dimeric enzyme (Table I). The subunit is inactive both at the alkaline pH used for the dissociation as well as at neutral pH.

Attempts to reconstitute active collagenase from its subunits. Incubation of subunit (2 mg/ml), which had been isolated as described above, in 5 mM Tris · HCl buffer at pH 7.0 in the presence of 10^{-5} M $ZnCl_2$ or in the presence of different concentration of $CaCl_2$ (from 5 mM to 0.5 M) did not restore any enzymic activity.

Influence of chelating agents on collagenase dissociation. In order to ascertain the role of Zn and Ca ions in the quaternary structure of *Achromobacter* collagenase, we have incubated the enzyme with chelating agents. In 0.1 M EDTA

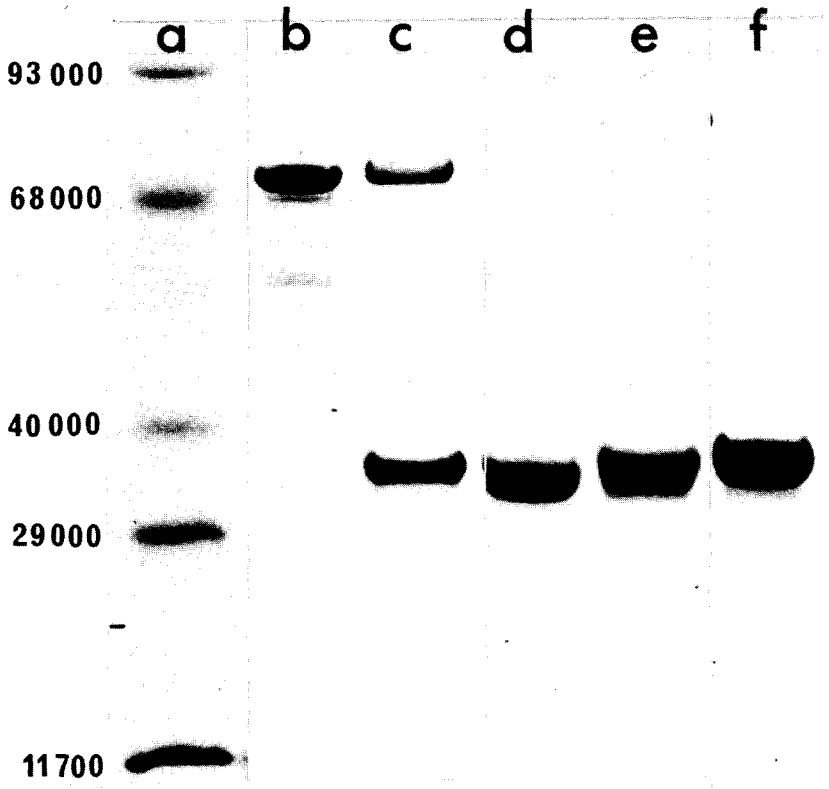

Fig. 1. SDS electrophoresis of *Achromobacter* collagenase (batch A) and of its dissociation products. (a) Standard mixture: Phosphorylase B (93 000), bovine serum albumin (68 000), aldolase (subunit) 40 000), carbonic anhydrase (29 000), cytochrome *c* (11 700). (b) Collagenase isolated from batch A. (c) Enzyme after 2 h of incubation at pH 10.5 in 0.1 glycine buffer containing 0.28 M LiCl. (d) Collagenase from batch A dissociated into subunit after 16 h in the same conditions as in (c). (e) The same subunit after pyridylethylation. (f) Enzyme after treatment with 0.1 M EDTA at pH 7.0.

at pH 7.0 and 20°C, the collagenolytic activity completely disappeared immediately after addition of the inhibitor. SDS electrophoresis of the incubation mixture shows (Fig. 1) that, after treatment with EDTA for 1 h, the dissociation of the inactive apoenzyme is complete. The apparent molecular weight of the dissociated subunits is 35 000, as had been found under the conditions of alkaline dissociation. After the treatment of the *Achromobacter* collagenase with 0.1 M EDTA at pH 7.0 the value of the sedimentation coefficient was $s_{20,w} = 2.9$.

In constrast to the action of EDTA, SDS electrophoresis showed that the treatment of *Achromobacter* collagenase with 5 mM *o*-phenantroline at pH 7.0 and 20°C decreases the enzymic activity to 1.5% without dissociation into monomers.

Pyridylethylation of the collagenase subunit. The subunit of molecular weight 35 000 obtained from the dissociation of the enzyme by alkaline treatment was reduced and the four cysteine residues were substituted with vinylpyridine as described in Materials and Methods. The results on Fig. 1 and in

TABLE I

AMINO ACID COMPOSITION OF *ACHROMOBACTER* COLLAGENASES AND THEIR SUBUNITS

Values are residues per mol.

Amino acid	Collagenase M_r 70 000	Subunit M_r 35 000	Pyridylethyl- M_r 35 000	Collagenase M_r 80 000	Subunit M_r 35 000	Peptide M_r 5 000
Half-cystine	7.9	3.8	0	8.0	4.0	n.d.
Aspartic acid	76.0	38.0	37.8	89.6	38.0	7.2
Methionine	n.d. *	2.9	2.8	5.8	2.7	0
Threonine	50.0	24.0	22.3	53.0	24.0	2.1
Serine	54.0	27.3	26.8	62.6	26.9	3.3
Glutamic acid	87.0	43.0	39.0	98.0	43.5	7.0
Proline	13.9	7.0	7.4	16.4	7.3	1.0
Glycine	54.2	27.1	27.6	65.0	27.0	4.6
Alanine	52.4	26.3	25.8	55.8	25.8	3.6
Valine	37.5	18.2	18.2	46.5	20.2	2.0
Isoleucine	30.8	14.2	13.4	32.2	14.2	1.8
Leucine	48.0	25.0	24.8	50.4	22.7	2.7
Tyrosine	34.8	15.5	15.2	34.1	15.0	4.6
Phenylalanine	30.0	14.8	12.4	30.2	12.8	2.6
Lysine	20.0	10.5	12.1	26.3	12.0	1.0
Histidine	16.4	8.4	7.6	17.4	8.0	0.9
Arginine	18.0	9.0	8.9	20.9	8.4	1.0
Tryptophan	16.3	8.6	n.d.	13.4	n.d.	n.d.
Pyridylethyl-cysteine			3.5			

* n.d., not determined.

Table I demonstrate that the reduction and pyridylethylation of the subunit change neither its molecular weight nor amino acid composition; it therefore consists of a single polypeptide chain.

N-terminal sequence. 15 mg of pure collagenase of M_r 70 000 were subjected to sequence analysis. A unique sequence of nine N-terminal amino acid residues was determined unambiguously: Thr-Ala-Ala-Asp-Leu-Glu-Ala-Leu-Val. This leads to the conclusion that the enzyme sample used in the study was homogeneous and that the two subunits are identical, at least in the N-terminal part of the polypeptide chain.

B. Achromobacter collagenase of M_r 80 000

Collagenase of specific activity 1.64 μkat · mg^{-1} which had been isolated from batch B and found to be electrophoretically homogeneous gives by gel filtration on SDS electrophoresis under the conditions which were used for the collagenase from batch A, a value for the apparent molecular weight of 80 000. When this enzyme was incubated at 4°C with 0.28 M LiCl at pH 10.5 the collagenolytic activity completely disappeared within the first minutes of incubation. After treatment for 2 h, the SDS electrophoresis showed the presence of three bands corresponding to molecular weights of 80 000, 40 000 and 35 000 (Fig. 2). Incubation for 16 h led to a total dissociation into inactive subunits of the same molecular weight, 35 000 as in the case of collagenase of M_r 70 000 (Fig. 2). When the incubation mixture was subjected to gel filtration on Sephadex G-100 column, a peak corresponding to a product of a molecular weight less than 8000 appeared in addition to the main peak of apparent molecular

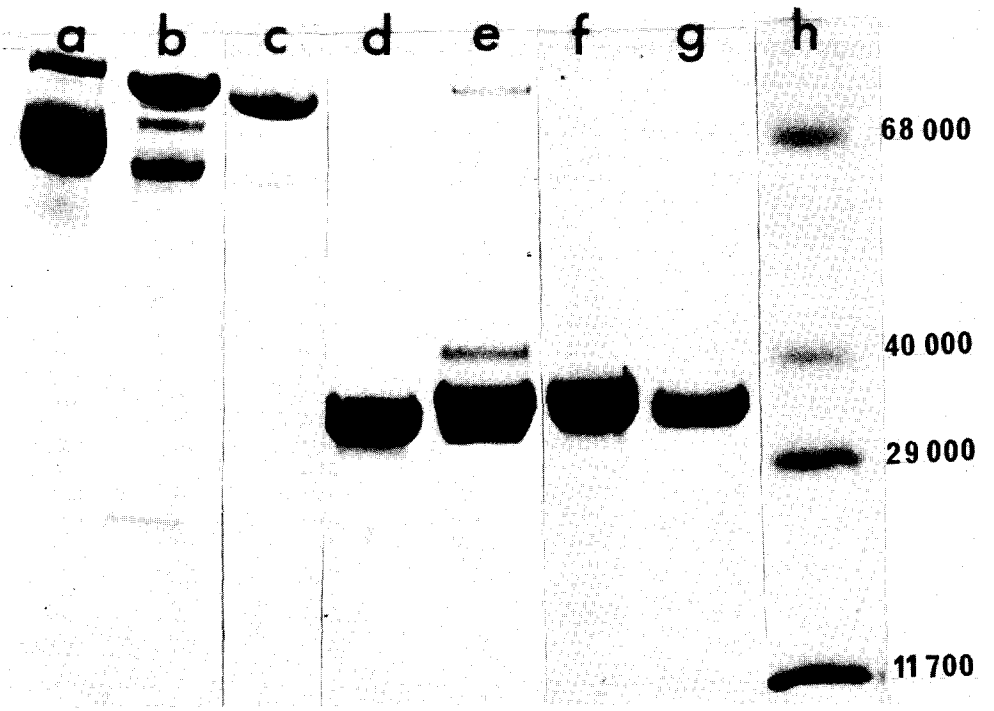

Fig. 2. SDS electrophoresis of *Achromobacter* collagenase (from the batch B) and of the products of its dissociation. (a) Standards: Phosphorylase B (93 000), bovine serum albumin (68 000). (b) Standards: Aspartokinase I-homoserine dehydrogenase I (86 000) and bovine serum albumin (68 000). (c) Collagenase from batch B. (d) The same collagenase after treatment with 0.1 M EDTA at pH 7.0 (e) Enzyme after 2 h of incubation at pH 10.5 in 0.1 glycine buffer containing 0.28 M LiCl. (f) Enzyme after 16 h of incubation at pH 10.5 in 0.1 M glycine buffer, containing 0.28 M LiCl. (g) The subunit after dissociation of collagenase (70 000) from batch A. (h) Standard mixture: Bovine serum albumin (68 000), aldolase (subunit 40 000), carbonic anhydrase (29 000), cytochrome *c* (11 700).

weight 35 000 (Fig. 3). This yellow coloured low molecular weight peptide is absent on the elution diagramme of 70 000 collagenase which was dissociated and gel filtrated under the same conditions. The low molecular weight peptide is not susceptible to staining after SDS electrophoresis.

From the difference between the value of the intermediate product of dissociation (40 000) and that of the completely dissociated subunit (35 000) its molecular weight can be estimated to be 5000. When the molecular weight of this peptide was determined by analytical Bio-Gel P-10 chromatography in 6 M guanidine HCl, the value of 4600 ± 10% was obtained.

The concentration and desalting of the 35 000 subunit was performed by ultrafiltration in an Amicon apparatus with the membrane UM-10. The low molecular weight fraction was concentrated and desalted on Biogel P-2 column. Both fractions were lyophilized.

The amino acid compositions of collagenase M_r 80 000 as well as of both products of its alkaline dissociation, the subunit and the peptide, are presented in Table I. The amino acid composition of the subunit is the same as that of the subunit from the collagenase of molecular weight 70 000. The sum of the

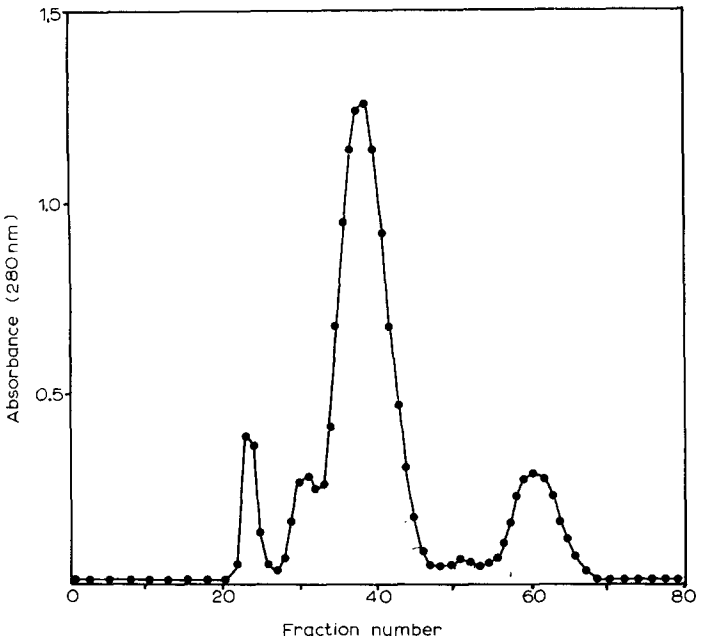

Fig. 3. Elution pattern of dissociated collagenase from batch B on Sephadex G-100 column. The column (69 × 2.2 cm) was equilibrated and eluted with 0.1 M glycine 0.28 M LiCl, pH 10.5 buffer. The flow rate was 25 ml/h, 3.8-ml fractions were collected. 40 mg of collagenase were incubated in 6 ml of the same buffer at 4°C. After 2 h the sample was applied on the column.

amino acid residues of the subunit and of the peptide corresponds to the half of the values found for the enzyme of M_r 80 000.

Therefore, in the collagenase of molecular weight 80 000 each subunit of 35 000 is strongly associated with a polypeptide of an approx. molecular weight of 5000. The interaction between the subunit and the polypeptide is so strong that it is not influenced by pretreatment of the sample for the SDS electrophoresis in the presence of 0.1% sodium dodecyl sulphate by heating together with 3% sodium dodecyl sulphate and 1% mercaptoethanol.

Discussion

The previously reported value of 104 000 for the molecular weight of collagenase from *A. iophagus* which was obtained in our laboratory, was deduced only from the zinc content [8]. It was close to the values of 111 700 and 106 900 which were found earlier by Welton and Woods [6] by electrophoresis in sodium dodecyl sulphate and gel filtration respectively. Our present study on the quaternary structure of this enzyme prompted us to reinvestigate first of all the values of the molecular weight reported earlier. The results presented here demonstrate the existence of two homogeneous forms of *Achromobacter* collagenase which differ in their molecular weights (70 000 and 80 000) and amino acid composition (Table I).

The molar activities of both forms are practically the same, 140 kat and 131 kat, respectively. The dissociation of both collagenases under alkaline con-

ditions in the presence of LiCl or at pH 7.0 in the presence of EDTA results in the appearance of an inactive subunit of the same molecular weight, 35 000, and the same amino aicd composition (Table I). Simultaneously, a peptide of M_r 5000 is liberated from the enzyme of M_r 80 000. In this case, after a rapid loss of activity the dissociation goes through an intermediary form of M_r 40 000; the subunit of M_r 35 000 and the peptide of M_r 5000 are the final products of the dissociation.

The peptide which is firmly held in the collagenase of M_r 80 000 was isolated by gel filtration after dissociation. Its amino acid composition (Table I) indicates that it is rich in aspartic and glutamic acids as well as in glycine and tyrosine. The question whether this subunit-peptide association is specific or whether it reflects a general ability of the enzyme to non-covalent interactions remains open. The high affinity for complex formation with collagen and different tissue components [18], products of substrate cleavage [19] or with natural polypeptidic inhibitors [20] was found for both vertebrate and bacterial collagenases. The ability of collagenase to bind certain peptides could explain the difference in the molecular weight reported in this work and the value of 106 000—111 000 for the same enzyme isolated earlier from a culture medium of different composition by Welton and Woods [6]. It should be emphasized that the two types of bonding, subunit-peptide and dimer, are very tight in the case of *Achromobacter* collagenase. They are affected neither by sodium dodecyl sulphate under reducing conditions, nor by usual dissociating agents. The dissociation could be brought about only by means which proved to be efficient to dissociate the three-component system of procarboxypeptidase A [21], another Zn-metallo protein.

From these results it follows that the simplest form of the active enzyme is a dimer composed of two subunits of M_r 35 000 each. It is premature to decide whether the two subunits are identical, but the unique N-terminal sequence Thr-Ala-Ala-Asp-Leu-Glu-Ala-Leu-Val of collagenase makes this assumption plausible.

The study of several vertebrate collagenases gave examples of the existence of active oligomers, formed from active subunits, as in the collagenase from mouse bone (41 000 and 84 000) [22], human rheumatoid synovium (25 000 and 50 000) [23] or rabbit alveolar macrophage (45 000, 85 000 and 165 000) [24]. Nevertheless, it is difficult to decide on the size of the active form of the enzyme in cases where the molecular weights were determined under conditions favourable to oligomer-monomer transition, as in alkaline buffers, in the presence of sodium dodecyl sulphate, EDTA, different salt concentrations. It cannot be entirely excluded that in the case of the collagenases which could be easily dissociated even at neutral pH and high salt concentration reversible dimerisation may take place after the salt concentration has been decreased for the enzymic assay. Therefore, it is difficult to find out which molecular form is really active.

The dissociation of another bacterial collagenase, from *C. histolyticum*, into four inactive subunits (25 000) after treatment of the enzyme with EDTA at pH 11 or without EDTA at pH 12 was reported by Levdikova et al. [25]. On the other hand, Lwebuga-Mukasa et al. [3] do not accept the subunit structure of *Clostridium* collagenase. The authors have obtained the same values for the

molecular weight of the enzyme by ultracentrifugation and in polyacrylamide gel electrophoresis in the presence of SDS and were led to the conclusion that different *Clostridium* collagenases are composed of a single polypeptide chain of M_r 72 000 or 81 000.

When bacterial collagenases from *Clostridium* [25] and *Achromobacter* are dissociated into subunits, this dissociation is accompanied by loss of enzymic activity. On the other hand, the dimeric form of the *Achromobacter* enzyme, firmly held by non-covalent bonds, has the highest collagenolytic activity yet known.

In this respect it seems of interest to recall the hypothesis of the action of vertebrate collagenases advanced by Brown et al. [26] according to which two recognition sites of collagen molecule could be simultaneously attached to identical subunits in a dimerized form of collagenase. The authors have postulated that a mammalian collagenase, like the DNA restriction enzyme, may be active in the dimeric form and that it recognises its substrate site by a similar two-fold symmetric arrangement of imino acid residues.

The biological function of vertebrate and bacterial collagenases is definitely different: The role of vertebrate collagenases is to contribute to the regulation of the equilibrium between native and degraded collagen in tissues, whereas the bacterial collagenases have to assure the rapid degradation of collagen in invaded tissues. This, together with the previous observations on the differencies in the specificity of vertebrate and bacterial collagenases on native collagen and synthetic substrates in vitro, led to a tendency to consider the two groups of these enzymes quite separately. However, advancing knowledge on the structure and specificity of these two groups opens the way to reconsider eventual similarities in their structural and functional relationships. New data are available now on the existence of subunits, on the role of the metal atom in the active site, on the ability of vertebrate collagenases to split synthetic substrates [27] as well as on the preferential cleavage of native collagen in its "weak" region by the bacterial *Achromobacter* collagenase in an analogous way to the vertebrate collagenases (Lecroisey and Keil, unpublished data, prepared for publication). In the view of these structural and functional analogies one can suggest that the difference in the biological role of vertebrate and bacterial collagenases may find expression in the dimer-monomer equilibrium of subunits possessing an analogous active site.

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