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CELL-SURFACE COLLAGEN-BINDING PROTEIN IN THE PROCARYOTE *ACHROMOBACTER IOPHAGUS* *

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Collagen and its high-molecular-weight fragments specifically induce an extracellular collagenase (EC 3.4.24.8) in the Gram-negative *Achromobacter iophagus*. During the induction process the inducer is concentrated on the bacterial outer membrane. Two-dimensional electrophoresis of ^{125}I-labelled outer membrane proteins has shown that, in particular, the amount of one protein which is already present on the surface of non-induced bacteria increases quantitatively when the inducer is added. After ^{125}I-labelling of the cell membrane and its solubilization, the same protein is retained selectively on a gelatin-Sepharose column. It has isoelectric point of 4.9–5.1 and molecular weight of 40 000. This molecular weight is close to that of the 35 000 of the collagenase subunit. However, their non-identity was proved in three independent ways: upon two-dimensional electrophoresis, only those proteins in the range corresponding to the collagenase dimer (M_r 70 000–80 000) react with fluorescent anticollagenase antibody system, whereas the spot of the collagen-binding protein (M_r 40 000) is negative; the solubilized collagen-binding protein is not retained by anticollagenase-Sepharose affinity chromatography; in vivo, it is not protected by anti-collagenase antibodies against lactoperoxidase iodination. A hypothesis for the possible role of the collagen-binding protein in the induction of collagenase is proposed.

Introduction

Achromobacter iophagus is a non-pathogenic aerobic Gram-negative bacterium first described by Woods et al. [1] **). This strain produces a collagenase (EC 3.4.24.8) which is a zinc metalloproteinase with high specific activity on native collagen and related substrates [3–5]. The active form is a dimer of molecular weight 70 000, composed of two subunits [6] with some structural features in common with thermolysin [7–9].

In 1976, we showed [10] that the synthesis of this collagenase is induced by collagen or its high-molecular-weight fragments, whereas the low-molecular-weight collagenase substrates or collagen fragments resulting from degradation of collagen by collagenase are without effect: both high molecular weight and the presence of intact peptide bond sensitive to the collagenase action must be present in the inducer. In a comparative study undertaken with a series of 30 other globular and fibrilar proteins, no effect of induction similar to that observed with collagen has been found (Emod and Keil, unpublished data).

Several independent studies have shown that

* This work is dedicated to the memory of Prof. F. Sorm.

** More recently it was proposed that *A. iophagus* is identical to *Vibrio alginolyticus* [2]. However, detailed bacteriological and biochemical characterization of the two strains has demonstrated constitutional and metabolic differences (unpublished data). As the nomenclature is not yet stabilized, the original designation '*Achromobacter*' is maintained in this paper.

the synthesis of collagen-degrading enzymes is dependent on the presence of collagen or its degradation products in the culture medium. Thus, this kind of stimulation of a 'gelatinase' was observed by Castaneda-Agullo in *Serratia marcescens* [11] and by Tanaka and Iuchi in *Vibrio pseudohaemolyticus* [12]. Two other collagenases were found to be induced by collagen and its degradation products: Dreisbach and Merkel [13,14] demonstrated in 1978 the induction of a collagenase in *Vibrio* B 30 by both collagen and byproducts resulting from the degradation of collagen by collagenase. Later Monboisse et al. [15] also observed collagenase induction in *Acinetobacter*. It is interesting to note that Biswas et al. [16] described a stimulating effect of collagen on collagenase synthesis in fibroblasts and synovial cells.

The peculiarity of *Achromobacter* collagenase synthesis is that only large molecules which have not been degraded by collagenase act as inducers. Data available from the study of Gram-negative bacteria have shown that molecules of molecular weight above 900 (peptides, oligosaccharides) cannot diffuse through the barrier of the outer cell membrane [17,18]. Transport of larger molecules (colicins, iron complexes, vitamin B-12, maltodextrins) from the outer space to the periplasm is mediated by a specific surface receptor [19–21]. This suggested that the pathway of collagenase induction may not follow the classical mechanism of enzyme induction by small ligands which was established in procaryotes [22,23].

The aim of this work was to search after the mechanism of inducer recognition by the cell. It produces proof for the existence of a protein in the outer membrane of *A. iophagus* which has specific affinity for collagen but which is structurally different from collagenase.

Materials and Methods

Materials. Homogeneous *Achromobacter* collagenase of specific activity 1.8 μkat/mg was obtained from crude collagenase (Institut Pasteur Production, Paris, France) by chromatography on DEAE-cellulose and Sephadex G-100 as described previously [5]. Salt-soluble rat skin [^{14}C]collagen, prepared according to Kang et al. [24] had a spec. act. of 10765 cpm/mg. A solution of 1 mg [^{14}C]collagen per ml, prepared as described by Gross and Lapiere [25] was dialyzed against the culture medium. The synthetic collagenase substrate 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine dihydrate (Pz-Pro-Leu-Gly-Pro-DArg) was a product of Fluka (Buchs, Switzerland). Na^{125}I was supplied by Amersham-Searle (Arlington Heights, IL, U.S.A.). Lactoperoxidase and glucose oxidase were from Sigma (St. Louis, MO, U.S.A.). Gelatin AT 100 (Rousselot, Paris, France), was an enzymatic hydrolysate of denatured calf skin collagen of average M_r 7000, exhaustively dialyzed and lyophilized. Calf skin gelatin type III was from Sigma. Casamino acids (a vitamin-free acid-hydrolized casein) were purchased from Difco (Detroit, U.S.A.). CH- and AH-Sepharose 4B were the products of Pharmacia (Uppsala, Sweden).

Bacterial culture and growth control. The collagenase producing strain of *A. iophagus* (I. 029, Institut Pasteur) was isolated by Woods et al. [1]. The culture medium consisted of a 2.5% solution of casamino acids in 0.1 M Tris-HCl buffer which was 0.4 M in NaCl and 2 mM in $CaCl_2$ (pH 7.6). For induction experiments, the inducer (gelatin AT 100) was added to final concentration 0.25%. Bacterial growth was measured by absorbance at 600 nm (1 cm, spectrometer Zeiss MQ3). 3 ml of the bacterial suspension (A 4.0–5.0) was incubated in Pyrex tubes under aeration by compressed air (1.4 atm) at 29°C. Samples for growth measurements and assays were withdrawn at 1 h intervals.

Spheroplasts and membrane preparation. Bacterial membranes were isolated according to Kabir [26]: 10 ml of bacterial culture were centrifuged at 12000 × g, the sediment resuspended in 0.1 M Tris-HCl buffer (pH 7.8) (1 ml buffer for A_{600} 10.0 of the original culture) for 45 min at 4°C under agitation, diluted with 6 vol. of the same buffer and centrifuged 10 min at 12000 × g. The sediment was resuspended in 0.01 M Tris-HCl buffer (pH 7.8) made 0.75 M in saccharose (0.7 ml for A_{600} 10.0 of the original culture) and incubated with crystalline lysozyme (0.1 mg per ml of suspension) 2 min at 0°C. After dilution with 2 vol. 1.5 mM EDTA, the mixture was incubated for 3 h at 4°C and the suspension of spheroplasts obtained by this treatment was lysed by pouring in 4

vol. ice/water under mild agitation. The membrane fraction was obtained by centrifugation at 48 000 × *g* for 30 min.

Assay of collagenolytic activity. Collagenase activity in the culture samples was determined colorimetrically using Pz-Pro-Leu-Gly-Pro-DArg according to Wünsch and Heidrich [27]. Data were recalculated on the basis of 1 μkat = 1 μmol substrate/s = 90 000 units according to Ref. 27.

Testing of anticollagenase polyclonal antibodies. Rabbit antibodies prepared against pure *Achromobacter* collagenase were purified by ammonium sulphate precipitation and chromatography on DEAE-cellulose (a gift of Dr. B. Bizzini). The stock solution contained 57.4 mg protein per ml. Immunodiffusion tests were carried out in 1% agar in 0.15 M NaCl. Anticollagenase at 10- to 160-times dilution gave single precipitin lines against pure *Achromobacter* collagenase (1 mg/ml solution in distilled water). The same was found for the collagenase subunit.

Labelling with ^{125}I [28]. Aliquots withdrawn from bacterial culture were diluted to 1 ml with 0.1 M Tris-HCl buffer (pH 7.6), which was 0.4 M in NaCl and 2 mM in $CaCl_2$, to give a final A_{600} of 5.0. Then 1.5 ml 5 mM glucose in the same buffer were added. After addition of 50 μl 10^{-5} M KI, 10 μl lactoperoxidase (677 units/ml), 1 mCi $Na^{125}I$ and 10 μl glucose oxidase (277 units/ml) the samples were incubated for 20 min, then centrifuged at 12 000 × *g* for 15 min. The precipitate was washed twice with 10 ml of the same buffer which was 150 mM in KI, lysed with 150 μl lysis Ampholine buffer [29] containing 9.5 M urea, 2% Nonidet P-40 and 5% 2-mercaptoethanol, and submitted to two-dimensional electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis (two-dimensional electrophoresis). Electrophoresis on slab gels containing 10% acrylamide and 0.17% bisacrylamide was performed according to O'Farrell [29]. The standard mixture used in the second direction contained β-galactosidase, phosphorylase, bovine serum albumin, aldolase (subunit) and chymotrypsinogen (M_r 130 000, 96 000, 40 000 and 25 000, respectively). Gels were stained with Coomassie brilliant blue and dried. Autoradiographs were prepared with Kodak X-ray film. For pH measurement of the first-dimension gel, a control gel after isoelectric focusing was cut into 5-mm slices which were then incubated with 1 ml distilled water overnight at room temperature, and pH was measured with Metrohm Herisau pH meter.

Transfer of proteins from two-dimensional electrophoresis on nitrocellulose sheets and immunochemical localization of collagenase. Proteins separated by two-dimensional electrophoresis were transferred from the gel to sheets of nitrocellulose (Schleicher-Schüll, Dassel, F.R.G.) according to Bowen et al. [30]. After protein transfer the nitrocellulose sheet was immersed for 2 h at 20°C in binding buffer A (10 ml Tris-HCl 3% bovine serum albumin/0.15 M NaCl (pH 7.4)), and washed with clearing buffer B (10 mM Tris-HCl/0.15 M NaCl (pH 7.4)). Afterwards the sheet was incubated subsequently in rabbit anticollagenase (diluted 1 : 50 with binding buffer A), in clearing buffer B, in fluorescent sheep anti-rabbit-IgG antiserum and then in clearing buffer B. Fluorescent spots on the dried sheet were observed in 240 nm Mineralight range.

Affinity chromatography on gelatin-Sepharose. 1.5 g CH-Sepharose 4B, washed as recommended by the producer, gave 4.5 ml gel; to this was added 1 g calf skin gelatin type III dissolved in 10 ml H_2O. The pH was adjusted to 5.0 and 0.192 g *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide-HCl (Sigma) in 10 ml H_2O was added at 20°C dropwise under gentle agitation; pH 5.0–5.5 was maintained during the first hour of reaction by dilute NaOH. After 16 h the slurry was washed by 1 M NaCl and distilled water. Amino acid analysis indicated that 5.7 mg ligand was bound per ml gel.

The sample corresponding to *A* 10.0 at 600 nm was withdrawn from a culture of *A. iophagus* which contained neither inducer nor a measurable collagenolytic activity, at the beginning of the stationary phase of growth. The surface proteins were labelled with ^{125}I as described above and the cells were lysed with 300 μl lysis Ampholine buffer [29]. The sample was then completed to 3 ml with 3.2 mM phosphate buffer (pH 7.3) which was 0.2 M in NaCl and 1 mM in EDTA, and centrifuged at 12 000 × *g* for 15 min. The supernatant was applied on a gelatin-Sepharose column (1 × 5 cm) which was equilibrated with the same phosphate buffer as was used for sample dilution. The column was washed at flow rate 15 ml/h with 100 ml

equilibration buffer followed by 8 M urea (pH 7.3). The fraction eluted with 8 M urea was dialysed against 5 l distilled water, lyophilized and analyzed by two-dimensional electrophoresis.

Affinity chromatography on anticollagenase-Sepharose. Polyclonal anticollagenase antibodies were coupled to AH-Sepharose 4B in a manner analogous to that described above for Gelatin-Sepharose. The final product contained 2.3 mg protein per 1 ml Sepharose. The chromatography on anticollagenase-Sepharose was done under the same conditions as described above.

Results

Achromobacter iophagus, when grown in the buffered medium of casamino acids for 7 h, does not produce extracellular collagenase (Fig. 1). This result confirms previous observations [10]. In a simultaneous experiment, addition of the inducer after 3 h of culture caused the appearance of collagenase in the medium. Samples of the bacterial suspension were taken 30 min before and 2 h after the addition of the inducer (Fig. 1a, b), the cells were washed, labelled by ^{125}I and analyzed by two-dimensional electrophoresis. The patterns on the right-hand side of Fig. 1 show that when the inducer is present in the culture medium (Fig. 1b), the relative amounts of three ^{125}I-labelled proteins, 7, 8 and 9 of M_r 20000, 38000 and 40000, respectively, increase towards the stationary phase of growth. For quantitative evaluation, the spots of proteins 1–10 were cut out from the gel and measured for ^{125}I (Table I). In particular, the amount of protein 8 (M_r 40000) increased almost

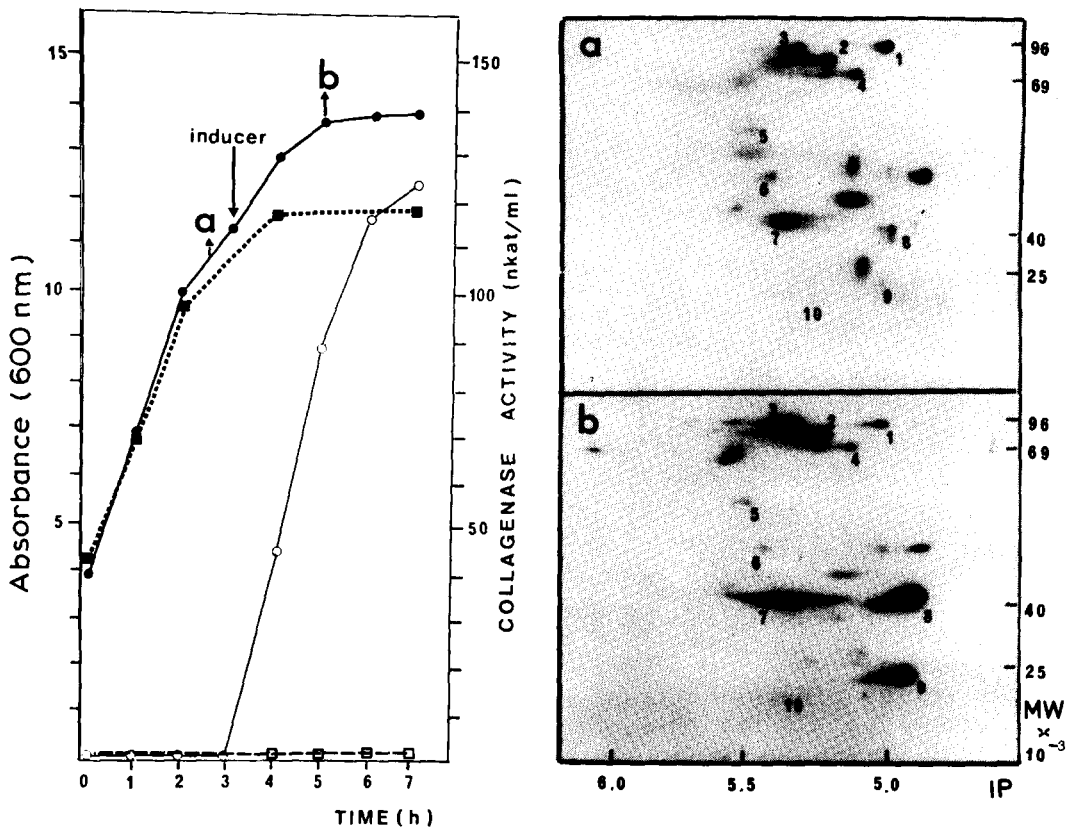

Fig. 1. Changes in outer cell membrane proteins during the induction of collagenase in the culture of *A. iophagus*. Left: cell growth (■- - - - -■) and extracellular collagenase activity (□- - - - -□) in absence of the inducer; cell growth (●———●) and enzyme activity (○———○) in culture to which inducer was added (arrow). Right: Two-dimensional electrophoresis of ^{125}I-labeled surface proteins of cells withdrawn from the culture before (a) and after induction (b); 1–10, arbitrary notation of protein spots.

TABLE I

QUANTITATIVE COMPARISON OF RADIOIODINATED CELL-SURFACE PROTEINS IN NON-INDUCED AND INDUCED CULTURES OF *A. IOPHAGUS*

	Culture: Non-induced	Induced	Ratio
	Fig: 1A	1B	1B/1A
Protein	cpm ($\times 10^{-3}$)	cpm ($\times 10^{-3}$)	
1	215	144	0.7
2	181	322	1.8
3	197	506	2.6
4	146	104	0.7
5	163	98	0.6
6	150	77	0.5
7	349	1390	4.0
8	202	1796	8.9
9	153	768	5.0
10	86	87	1.0

9 times. If cells were growing in the presence of casamino acids only, the amount of proteins 7, 8 and 9 did not increase significantly.

The content of collagen in membranes and cytoplasm during the induction process was measured in bacteria induced by [^{14}C]collagen; the cells were transformed into spheroplasts by lysozyme treatment and lysed in hypotonic medium and the membranes and cytoplasm were separated as described in Methods. The distribution of ^{14}C label is given in Table II.

In order to determine whether any of the cell-surface proteins have an affinity for collagen-like structure, the following experiment was undertaken. A 1 ml sample was withdrawn at 2.5 h intervals (Fig. 1a); it was free of collagenolytic activity. The cells, labelled by ^{125}I, were lysed and diluted as described in 'Methods' and submitted to chromatography on a gelatin-Sepharose column. The fraction retained by the affinity column and eluted by 8 M urea was analyzed by two-dimensional electrophoresis (Fig. 2a), which shows that the major retained component is the protein 8 of M_r 40000, aside from traces of different minor ^{125}I-labelled proteins.

The affinity of the protein of M_r 40000 for gelatin as well as its molecular weight close to that of the collagenase subunit (35000) raised the question of whether it could be an inactive precursor of collagenase. The following experiments were therefore undertaken.

When the proteins separated by two-dimen-

TABLE II

INDUCER LOCALIZATION DURING THE INDUCTION OF *A. IOPHAGUS* BY [^{14}C]COLLAGEN

Incubation time (h)	Collagenase (nkat/ml)	[^{14}C]Collagen distribution (% of cpm/min)		
		medium	cytoplasm	membrane
0	0	100	–	–
2	2.2	98.4	0.02	1.40
7	41.1	96.5	0.09	3.41

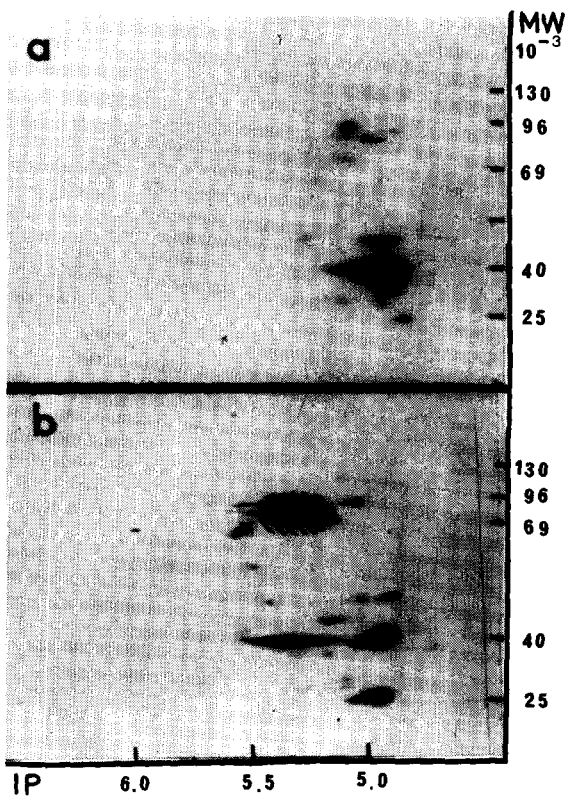

Fig. 2. Localization of collagen-binding protein and of anticollagenase-binding proteins by two-dimensional electrophoresis. (a) ^{125}I-labeled proteins bound and desorbed from gelatin-Sepharose affinity column. Major spot corresponds to protein 8 in Fig. 1; (b) anticollagenase-positive area (hatched) after transfer of cell proteins from two-dimensional electrophoresis (Fig. 1b) on nitrocellulose sheet and detection with fluorescent antibodies.

sional electrophoresis were transferred onto nitrocellulose sheets, it was possible to demonstrate which of them reacts with specific anticollagenase antibodies: the treatment with anticollagenase followed by binding of fluorescent sheep anti-rabbit-IgG antibodies revealed a specific interaction only in the range of M_r 70000–80000 (proteins 2–4), which corresponds to the molecular weight of collagenase dimer; on the contrary, no binding was observed in the M_r 40000 range (Fig. 2b).

In an independent experiment, *Achromobacter* culture was grown as described in Fig. 1a. At the beginning of the stationary phase of growth, polyclonal anticollagenase antibodies were added (final concentration 6 mg antibody protein per ml) and 1 h later the inducer was added to the final concentration of 1.5%. No traces of collagenolytic activity were observed under these conditions of culture. After 4 h, a 1 ml sample was withdrawn for lactoperoxidase ^{125}I labeling. The analyses by two-dimensional electrophoresis have shown that under the conditions described above the amount of collagen-binding protein 8 increased in the same way as shown in Fig. 1b. The presence of anti-collagenase, therefore, blocked the appearance of enzyme activity in the medium, but it neither influenced the relative increase of protein 8 on the cell surface, nor protected it against ^{125}I labeling.

When an aliquot of the same iodinated sample was lysed and applied on an anti-collagenase-Sepharose column under the same conditions as used for gelatin-Sepharose, the protein 8 of M_r 40000 was not retained, whereas *Achromobacter* collagenase under analogous conditions is retained. This indicates that there is no antigenic similarity between the protein 8 of M_r 40000 and collagenase.

Discussion

Our results show that on the surface of *A. iophagus* is present a protein (8), which binds in vitro selectively to denatured collagen (gelatin) (Fig. 2a). This protein has a molecular weight of 40000 and an approximate isoelectric point of about 5. It is labelled by ^{125}I in the presence of the lactoperoxidase-glucose-glucoseoxidase system, which assures the selective labelling of bacterial outer membrane proteins.

The facts that collagen concentrates on the surface during the induction and that protein 8 has an affinity for the high-molecular-weight inducer as well as that the content of protein 8 increases during the induction, suggest that it could play a role of a collagen receptor mediating the induction process.

Collagen receptors were already described in eucaryotes. The specific interaction of collagen and its fragments with the surface of fibroblasts was studied by Goldberg [31] and Chiang et al. [32]. In the last case, the interaction between receptor and collagen fragments was followed by structural changes which led to chemotactic migration. This receptor is different from LETS protein, which has also binding affinity for collagen [33].

Up to now, the presence of a collagen receptor in a procaryote has not been observed. However, in the case of *A. iophagus* it can be explained by the natural environment of the microorganism: this last is in contact with a high concentration of collagen during the infection of cow skin through which it spreads [1].

Previous studies on outer membrane proteins of Gram-negative bacteria have shown that receptors exist on the membrane even in the absence of a specific ligand. However, in its presence the amount of the receptor can increase to the point that it becomes a major cell-surface protein [34]. Protein 8, which binds specifically in vitro to denatured collagen, occurs on the surface of *Achromobacter* independently of the induction process. Nevertheless, its quantity increases considerably in the presence of high-molecular-weight fragments of collagen, which in turn are indispensible for the synthesis of collagenase (Fig. 1).

Both collagenase and collagen-binding protein 8 have specific affinity for collagen; the values of their molecular weights, 40000 for protein 8 and 35000 for collagenase monomer (7) are also close. Collagen-binding protein 8 could thus represent an inactive precursor of collagenase on the cell outer membrane. This possibility was eliminated by three independent approaches.

The transfer of proteins separated by two-dimensional electrophoresis followed by antibody fixation was relevant in this respect: even if the diffuse response did not allow to detect a single protein spot, the only group of proteins (2–4, Fig.

2b) which bind anticollagenase is in the range of 70 000–80 000, identical to the molecular weight of active collagenase. On the contrary, no binding was observed with protein 8.

As was earlier shown in our laboratory, the enzymic activity of *Achromobacter* collagenase is fully inhibited by its specific antibodies in vitro (Emod, unpublished data). In vivo, when bacteria grew in presence of both antibodies and inducer, the collagenase activity in the medium was absent; nevertheless, the accessibility of protein 8 for lactoperoxidase iodination was not abolished. If its structure were similar to the structure of collagenase, it would be most probably shielded by antibodies.

The lack of affinity of protein 8 for Sepharose-bound collagenase antibodies is an additional immunochemical evidence of its structural difference from collagenase.

A direct evidence that protein 8 is involved in the mechanism of collagenase induction is still lacking. A working hypothesis on the relationships between the inducer, surface receptor and induced enzyme would be the following: protein 8, which contains the binding site for the fixation of collagen or of its macromolecular fragments, represents or makes part of a surface receptor. The formation of inducer-receptor complex is the first step in the information transfer which proceeds through the periplasmic space to the cytoplasm, whereas collagenase synthesis and its transport to the outer space is the last step. The system is self-adjusting: active collagenase splits collagenase-sensitive bonds in the inducer-receptor complex. As was previously shown, collagen digested by collagenase loses its inducing properties [10]. The degraded inducer has consequently no more affinity for the receptor, the complex dissociates which, in turn, stops collagenase synthesis.

Further study of the macromolecular inducer-receptor-enzyme system in *Achromobacter* and closely related Gram-negative bacteria is under way.

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