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Biochemical Characterization of *Porphyromonas (Bacteroides) gingivalis* Collagenase

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A protease was purified from *Porphyromonas gingivalis* 1101, a clinical isolate, by sequential sodium dodecyl sulfate-polyacrylamide gel electrophoresis, substrate diffusion gel electrophoresis, and electroelution. The enzyme cleaved radiolabeled human basement membrane type IV collagen and the synthetic collagen peptide substrate for eukaryotic collagenases. It was inactivated by the thiol protease inhibitor *N*-ethylmaleimide but not by EDTA or EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and activated by reducing agents such as β-mercaptoethanol. The enzyme exists as an active precursor protein of molecular mass 94 kDa and undergoes proteolytic cleavage to 75-, 56-, and 19-kDa forms. Biotin-labeled collagen bound specifically to the 94-kDa form of the protein and to its cleavage products in ligand blots, suggesting a role for this enzyme not only in collagen degradation but also in adhesion to collagenous substrata.

Several studies implicate black-pigmented *Porphyromonas (Bacteroides) gingivalis* as an important etiological agent associated with the pathogenesis of periodontal disease. In the course of many bacterial infections, proteases are putative virulence factors. Examples include zinc metalloproteases from the insect pathogens *Serratia* spp. (18) and the fish pathogen *Vibrio anguillarum* (13) and a protease from *Legionella pneumophila* (1). Such proteases are essential either for the initial invasive stages of the infection or for protection against the host immune response. In human bacterial infections, inactivation of the host immune response by proteases has been demonstrated with the elastase from *Pseudomonas aeruginosa* (17) and the immunoglobulin protease from *Neisseria gonorrhoeae* (14).

The proteolytic nature of *P. gingivalis* strains has been well documented in the literature, where they have been shown capable of hydrolyzing gelatin (25), complement (20), and immunoglobulins A and G (7). Several proteases, including an intracellular, trypsinlike protease (23) and a glycyl-prolyl protease (5), have been isolated and characterized. Since collagen is the major constituent of the gingival connective tissues, perhaps the most important bacterial enzyme associated with the tissue destruction seen in periodontal disease is the collagenase. Although many workers have identified collagenase activity in various *P. gingivalis* strains, the biochemical nature of the enzyme has not been established. In this work, we report the purification and characterization of the *P. gingivalis* collagenase.

MATERIALS AND METHODS

Culture conditions. *P. gingivalis* was grown on blood agar plates in a gas mix containing 80% N_2, 15% CO_2, and 5% H_2 according to Greiner et al. (4).

Collagen labeling. Collagen was radiolabeled for the microplate assay (see below) with Iodogen (Pierce Chemical Co.) dissolved in chloroform and coated onto Eppendorf tubes (50 mg per tube) (8). The coated tubes were washed briefly in phosphate-buffered saline (PBS) prior to labeling experiments. Subsequently, 100 μg of collagen in PBS and 100 μCi of iodine-125 (Amersham) were added to the tube. The reaction was allowed to proceed for 10 min at room temperature, after which unincorporated ^{125}I was removed by desalting on a Sephadex G-25 column (Pharmacia) in PBS–0.5% Triton X-100. The amount of labeled protein was determined by trichloroacetic acid precipitation and gamma counting.

Microplate assay for collagenase. The influence of pH and the effect of protease inhibitors on collagenase activity were assessed by a microtiter assay done by the method of Robertson et al. (15). Briefly, the wells of polyvinyl chloride microtiter plates (Becton Dickinson) were coated with 30,000 cpm of radiolabeled collagen diluted in 50 μl of 0.06 M carbonate buffer, pH 9.6, per well, and the supernatant was allowed to evaporate overnight at 37°C. The wells were subsequently washed in PBS three times for 10 min each time, incubated in PBS for a further 2 h at 37°C, and washed as before. Duplicate 50-μl samples of *P. gingivalis* cell extract were added to the wells and incubated overnight at 25°C. Supernatants were aspirated from the plate, and individual supernatants and wells were counted in a liquid scintillation counter (Beckman). The background counts released by buffer alone were subtracted from each sample. Enzyme activity was determined over a range of pHs and with various protease inhibitors. *Clostridium histolytica* collagenase (Sigma) was used as a positive control.

Synthetic peptide cleavage. The specificity of the protease activity in *P. gingivalis* cellular fractions was assessed by the ability of the protease to cleave the synthetic collagen substrate 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-glycyl-L-isoleucyl-L-alanyl-L-glycyl-L-glutaminyl-D-arginine (Calbiochem) by the method of Masui et al. (9).

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using the Mini PROTEAN II gel system (Bio-Rad). Samples were

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solubilized in SDS sample buffer for 30 min at 37°C and then electrophoresed at room temperature.

Visualization of collagenase bands. After electrophoresis, the gels were placed in 2.5% Triton X-100–50 mM Tris HCl (pH 7.6) for 30 min at room temperature to remove the SDS and subsequently were washed several times in 50 mM Tris HCl (pH 7.6). Substrate diffusion gels were incubated in a 1% collagen solution for 1 h at 37°C to allow the substrate to diffuse into the gels. After being washed in 50 mM Tris HCl (pH 7.6), the gel was incubated at 37°C for 1 h more. Proteolytic digestion was visualized by staining the gels in Coomassie blue R-250 (Serva). After destaining, collagenase activity appeared as clear bands against a dark-blue background.

Biotin labeling of collagen and ligand blotting. Human basement membrane collagen type IV (Sigma) was labeled with *N*-hydroxysuccinimide biotin (Pierce Chemical Co.) as follows: *N*-hydroxysuccinimide biotin dissolved in dimethyl sulfoxide was added at a final concentration of 5 mM to 100 μg of collagen dissolved in PBS and incubated at room temperature for 2 h. Excess biotin was removed by overnight dialysis against PBS (three changes, 4°C), and the labeled collagen was stored at −20°C until use. For ligand blotting, SDS-PAGE proteins were transferred to nitrocellulose and then blocked for 1 h in PBS–0.1% Tween 20 prior to overnight incubation at 4°C with the biotin-labeled collagen diluted 1:500 in the buffer described above. The filter was subsequently washed three times in PBS–0.1% Tween 20 and incubated for 1 h with alkaline phosphatase-labeled streptavidin (1:2,000 dilution). After being washed, collagen bound to *P. gingivalis* proteins was visualized using nitroblue tetrazolium (1 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.5 mg/ml) as substrate in 100 mM Tris HCl (pH 9.6).

Electroelution of proteins after SDS-PAGE. Proteins to be electroeluted were identified by Coomassie blue staining, after which the appropriate bands were excised with a sterile scalpel blade and placed in water. After three washes of 10 min each, the gel strips were incubated in the electroelution buffer (25 mM Tris HCl [pH 7.5], 0.1% SDS) for 1 h. Electroelution was performed in an elution chamber (Bio-Rad) for 2 to 3 h at 10 mA per tube, after which the protein solution was removed and precipitated at −20°C overnight in 5 volumes of 100% ethanol. The precipitated protein was centrifuged (Sorvall HB4 rotor, 27,000 × *g*, 30 min, 4°C), dried, and suspended in SDS-PAGE sample buffer.

RESULTS

Cell fractionation. The *P. gingivalis* proteolytic activity was initially localized by solubilizing and fractionating cells grown for 4 days at 37°C under anaerobic conditions. The cells were sonicated in PBS and ultracentrifuged, and the supernatant was removed (total soluble [TS] fraction). The cell pellet was suspended in PBS containing 0.5% SDS and 5% β-mercaptoethanol and centrifuged as described above, and the supernatant was collected as the membrane fraction. These two fractions were analyzed by SDS-PAGE under reducing and nonreducing conditions, revealing several proteins specific for each fraction (Fig. 1). To assess which fraction contained collagenase activity, the samples were run on a polyacrylamide gel copolymerized with human basement collagen type IV and incubated for 1 h. The cell fractions were also able to cleave casein, as seen in substrate-PAGE (data not shown), but in order to specifically identify the collagenase and not proteases in general, all

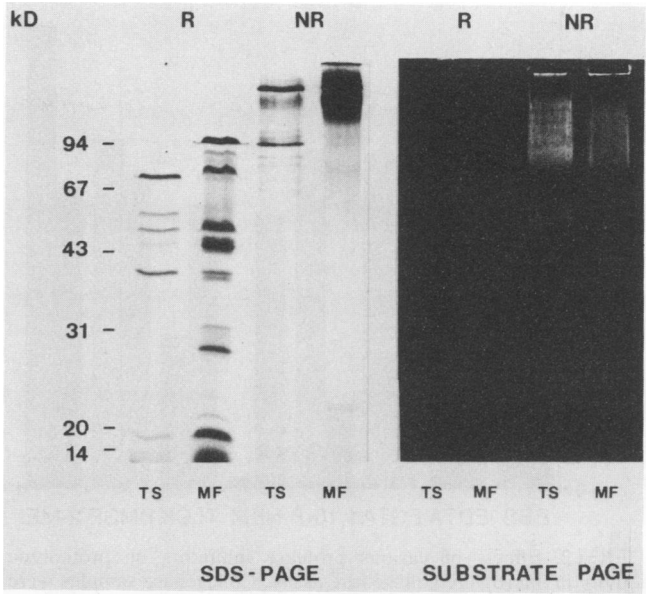

FIG. 1. SDS-PAGE analysis of *P. gingivalis* cell fractions. TS, total soluble fraction; MF, membrane fraction; R, reducing conditions; NR, nonreducing conditions. Approximately 10 μg of protein was loaded onto each lane. Left panel, SDS-PAGE; right panel, substrate-PAGE.

further studies used only collagen as substrate. After staining with Coomassie blue, light areas of proteolysis could be seen in both the TS and membrane fractions, but only under nonreducing conditions (Fig. 1, right panel). In both samples, the proteolytic activity was located at the top of the gel, while in the TS fraction, a second area of activity was observed in approximately the middle of the gel. Since the proteins were run under native conditions, i.e., without boiling or reduction, the proteins could only be assigned relative molecular weights in both the SDS-PAGE and substrate gels.

Inhibition of collagenase activity by various protease inhibitors. The putative collagenase was further characterized by using a microplate assay with native ^{125}I-labeled collagen as substrate. The pH optimum of the enzyme was assessed with a range of buffers of pHs 2 to 10. *C. histolytica* collagenase was used as a positive control. The pH optimum lay between pH 6 and 7 for both the *P. gingivalis* and the *C. histolytica* collagenases (data not shown). Differences, however, were observed when enzyme activity was assessed in the presence of various protease inhibitors. Those compounds which inhibit metalloproteases, e.g., EDTA, EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 1,10-phenanthroline, inhibited the *C. histolytica* collagenase between 75 and 95% but had limited effect on the *P. gingivalis* enzyme, with values of 28.8, 20.2, and 25%, respectively. TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone), a chymotrpysin inhibitor, and phenylmethylsulfonyl fluoride, specific for serine and thiol proteases, also had minimal inhibitory effects (29 and 14% inhibition, respectively). Thiol protease inhibitors, however, had a pronounced effect on the *P. gingivalis* enzyme: *N*-ethylmaleimide (NEM), with 98.2% inhibition, and TLCK (*N*-α-tosyl-L-lysine chloromethyl ketone), with 61% inhibition of enzyme activity. β-Mercaptoethanol caused a small but reproducible increase in activity (110%) compared with

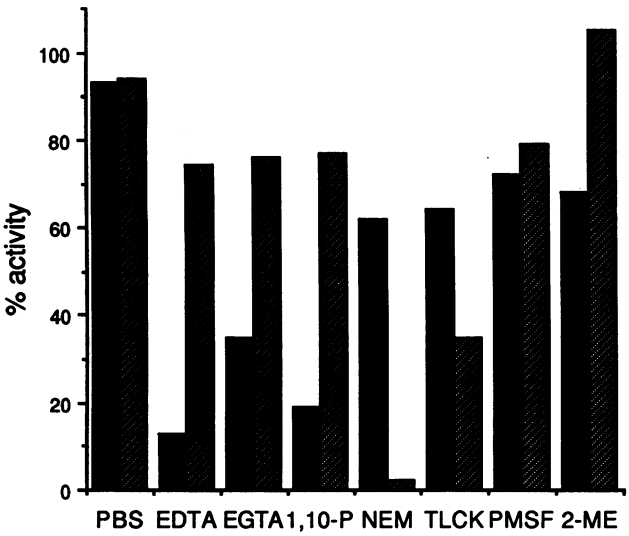

FIG. 2. Effects of various protease inhibitors on proteolytic activity in microplate collagenase assay. Collagenase samples were added to the wells containing collagen as substrate in the presence of inhibitors and incubated overnight at 37°C, after which both supernatant and well were counted separately. Solid boxes: *C. histolytica* collagenase; hatched boxes, *P. gingivalis* cell extract. Abbreviations and concentrations of inhibitors dissolved in PBS: EDTA, 10 mM; EGTA, 10 mM; 1,10-phenanthroline (1,10-P), 10 mM; NEM, 5 mM; TLCK, 1 mM; phenylmethylsulfonyl fluoride (PMSF), 10 mM; 2-mercaptoethanol (2-ME), 5 mM.

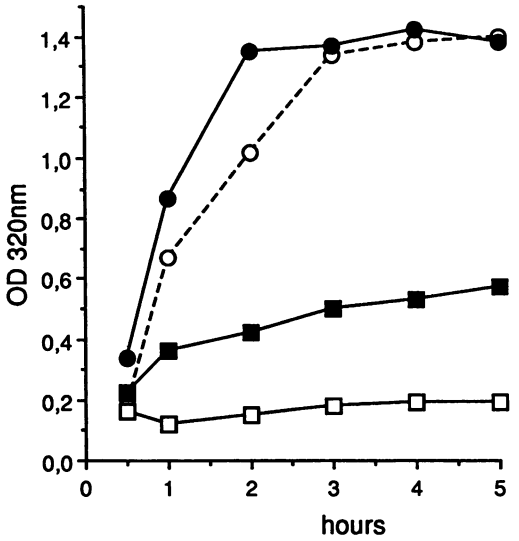

FIG. 3. Cleavage of synthetic collagen peptide by *P. gingivalis* TS fraction and *C. histolytica* collagenase in the presence and absence of inhibitors. Samples were removed at various times, the reaction was terminated by acidification, and the amount of cleaved peptide was measured at an optical density of 320 nm (OD 320 nm). Solid circles, *P. gingivalis* TS fraction; open circles, *C. histolytica* collagenase; solid squares, TS fraction with TLCK; open squares, TS fraction with NEM. Each time point represents the average of three experiments. NEM was used at a concentration of 5 mM, and TLCK was used at 1 mM. Negative controls included PBS alone and PBS with the respective protease inhibitor.

incubation in PBS alone (Fig. 2). Although several bacterial samples of differing growth stages (i.e., 3 to 5 days of growth on plates) were analyzed, no significant variation in either the cleavage rates or the amount of collagen degraded was detected.

Cleavage of defined collagen peptide. The ability of the protease to cleave a defined peptide encoding the cleavage site for eukaryotic collagenases was tested. Over a period of 5 h, more than 90% of the synthetic peptide was cleaved, with kinetics comparable to that of the commercially available *C. histolytica* collagenase. Cleavage by the *P. gingivalis* enzyme was inhibited by NEM (95%) and TLCK (68%) (Fig. 3) at levels comparable to those seen in the microplate collagenase assay with native collagen.

Collagenase purification by electroelution in SDS-PAGE. One problem encountered with gels copolymerized with substrate was that the resolution of the enzyme activity in the TS fraction was poor. To overcome this, the proteins studied were separated by nonreducing SDS-PAGE, after which the gel was placed in the substrate solution. Using such substrate diffusion gels, an exact correlation between a defined protein band in SDS-PAGE and the proteolytic activity observed in substrate diffusion gels could be made, thus allowing the individual proteolytically active proteins to be purified for further study.

The TS fraction was separated by 7.5% SDS-PAGE, and the two main proteolytic bands were identified after substrate diffusion and then excised and electroeluted. After ethanol precipitation, the samples were analyzed under reducing conditions by SDS-PAGE. Neither of the proteolytic bands eluted from the substrate diffusion gel could be detected, but instead, several lower-molecular-weight bands were present. Both proteolytic bands, however, gave the same pattern under reducing conditions, suggesting that they were somehow related (data not shown). This implied that the bands observed in the original gels contained either a tightly bound complex of proteins or fractions of one protein.

Proteins made up of subunits are readily dissociated by the addition of various concentrations of urea (2). Incubation of the native *P. gingivalis* TS fraction with urea followed by SDS-PAGE revealed slight changes in the migration pattern of several proteins but not in the two protease bands or in their proteolytic profiles in substrate diffusion gels (data not shown). This raised the possibility that the observed proteins were being degraded or processed into smaller components either by a contaminating protease or by autoproteolysis during the electroelution procedure.

Inhibition of autoproteolysis by acidification. If the collagenase was undergoing autoproteolysis, then inactivation of the enzyme might prevent this from happening. When the microplate assay was used, no collagenase activity was detected at pH 2. These conditions were then used to inactivate the protease. HCl was added to the TS fraction to an end concentration of 0.1 M, and then the fraction was dialyzed against 1 mM HCl overnight at 4°C. The proteins were separated by SDS-PAGE and identified by substrate diffusion, and the larger, more-active band was electroeluted, precipitated, and analyzed as before. The larger band now remained stable, even after being boiled for 10 min in 1% SDS–5% β-mercaptoethanol, with only a small amount of the 75-kDa product detectable (Fig. 4, lane 2). The molecular mass of the inactivated protease was 94 kDa. The protease was partially inactivated by dialyzing the TS fraction against 1 mM HCl for only 4 h. When this preparation was analyzed, defined cleavage products of 75, 56, and 19 kDa were seen, as was the 94-kDa precursor (Fig. 4, lane 1).

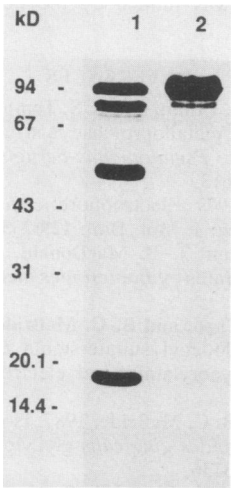

FIG. 4. Autoproteolytic activity of purified protease after incubation in 1 mM HCl for various times. Lane 1, partial inactivation of proteolysis achieved after 4 h of incubation; lane 2, total protease inactivation after 24 h of incubation. After HCl treatment, the samples were neutralized and analyzed by 10% SDS-PAGE under reducing conditions.

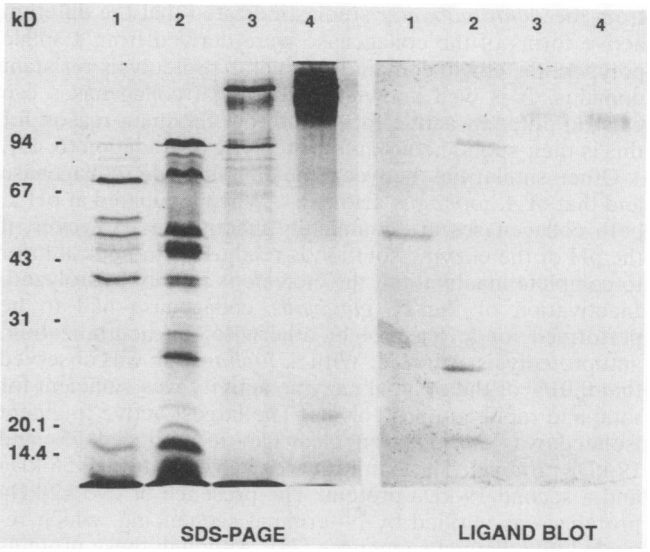

FIG. 5. Ligand blot of *P. gingivalis* proteins with biotin-labeled collagen. Samples were separated by 10% SDS-PAGE (left panel) and blotted onto nitrocellulose membranes (right panel). Binding of the labeled collagen was performed overnight at 15°C, and the bands were visualized by using alkaline phosphatase-labeled streptavidin. Lanes: 1, TS fraction, reduced; 2, membrane fraction, reduced; 3, TS fraction, nonreduced; 4, membrane fraction, nonreduced.

The 94-kDa protein binds collagen in ligand blots. *P. gingivalis* binds to spheroidal hydroxyapatite beads coated with collagen, and this binding may be an important means by which the bacteria adhere to and destroy collagen structures in the periodontum (12). To test if the *P. gingivalis* collagenase acts as the adhesin in this process, biotin-labeled collagen was used to probe Western blots (immunoblots) of the TS fraction. The collagen substrate bound to one protein of 56 kDa in the TS fraction and to three proteins of 94, 30, and 19 kDa in the membrane fraction under reducing conditions (Fig. 5, right panel, lanes 1 and 2). When samples were run under nonreducing conditions, the labeled collagen bound to two areas near the top of the gel that correlated exactly with the areas of highest proteolytic activity (Fig. 5, right panel, lanes 3 and 4) as seen in substrate PAGE (Fig. 1). A 94-kDa band was identified both by reactivity with the biotin-labeled collagen in the membrane fraction (Fig. 5, lane 2) and by the purification procedure.

DISCUSSION

Many clinical dental studies indicate that *P. gingivalis* is an important etiological agent in periodontitis. This pathogen produces a variety of proteases that differ in size, pH optima, sensitivity to inhibitors, ability to hydrolyze specific substrates, and location within or outside the bacterial cell. To date, several proteases have been isolated from various *P. gingivalis* strains; all of these proteases show unique specificities. When assayed by SDS-PAGE containing covalently bound bovine serum albumin, a total of eight distinct bands of proteolytic activity were detected with *P. gingivalis* strains ATCC 33277, HW11D-5, W83, and RB46D-1 (4). Each of these bands exhibited either a unique localization or a unique inhibition profile.

This work showed that the TS fraction isolated from *P. gingivalis* 1101 cleaved native collagen in both substrate diffusion gels and the microplate collagen assay. The pH optimum was comparable to that of the *C. histolytica* collagenase, although the inhibition profile was different. This is in agreement with the data from Mayrand et al. (10) and with earlier observations by Gibbons and MacDonald (3) that *Bacteroides melaninogenicus* produced an enzyme that was able to cleave collagen. The inhibition profile was initially surprising, since most collagenases are metalloproteases and not thiol proteases inhibited by NEM. The inhibitory effect of reducing agents such as dithiothreitol or 2-mercaptoethanol on collagenases is commonly observed with eukaryotic collagenases isolated from rat uterus tissue (26), human polymorphonuclear leukocytes (11), and gingival fluid (24). However, *P. gingivalis* collagenase activity was elevated with 2-mercaptoethanol. These features, apparently unique to the *P. gingivalis* collagenase, have already been noted in a study of collagenolytic activity in culture supernatants from 20 anaerobic bacteria (21).

To date, all collagenases isolated from eukaryotic or bacterial sources require Ca^{2+} ions for proteolytic action and are inhibited by various chelating agents. However, the *P. gingivalis* collagenase retained almost full activity in 0.05 M EDTA, suggesting that such ions were not required either for the very strong complex formation binding the enzyme to the collagen substrate or for full catalytic activity. Thus, although the *P. gingivalis* enzyme demonstrates the substrate specificity of a collagenase, its mode of action resembles that of a cysteine protease.

Another well-characterized bacterial collagenase is that from *Achromobacter iophagus*, which has two high-molecular-mass forms, A1 (110 kDa) and A2 (90 kDa), that can be isolated from the culture supernatant (22). The forms had identical C-terminal but different N-terminal sequences, although they exhibited the same activities towards synthetic substrates and collagen and had similar inhibition profiles. The original TS fraction from *P. gingivalis* also had two areas of proteolysis in substrate diffusion gels, and when both proteolytic bands were isolated and analyzed in SDS-PAGE under reducing conditions, they gave similar patterns, suggesting that they were related. The results obtained

from the *Achromobacter* studies indicated that the different active forms of the collagenase were derived from a single polypeptide chain containing several proteolysis-resistant domains. It is well known that bacterial collagenases can exist in different active forms and that the main reason for this is their spontaneous autoproteolytic degradation (6, 19).

Other similarities between the *P. gingivalis* collagenase and that of *A. iophagus* also exist. When incubated at pH 2, both collagenases are completely inactivated. However, if the pH of the enzyme solution is readjusted to neutral prior to complete inactivation, the enzyme is rapidly hydrolyzed. Inactivation of the *P. gingivalis* collagenase had to be performed for at least 24 h; otherwise, on neutralization, autoproteolysis occurred. With *A. iophagus*, it was observed that 0.01% of the original enzyme activity was sufficient for total and rapid autoproteolysis. The largest active fragment isolated was 94 kDa; on cleavage, it produced 75- and 19-kDa proteins. These in turn are cleaved to form a 56-kDa and a second 19-kDa protein. The presence of two 19-kDa proteins was verified by N-terminal sequencing, which revealed two distinct sequences (7a). Although these proteins bound collagen in ligand blots, they were devoid of catalytic activity in the substrate-PAGE assay used in this work. The possibility that the 19-kDa band represents a collagen adhesin is currently under investigation with more-refined assay methods.

Since collagen is the most important structural component of the periodontum (16), knowledge of bacterial interactions with it are important for understanding the mechanisms of tissue destruction during episodes of periodontal disease. The binding of biotin-labeled collagen to the 94-kDa protein, as observed in the ligand blotting experiments, is in agreement with the results of Naito and Gibbons (12), who demonstrated a similar binding of *P. gingivalis* cells to collagen-coated hydroxyapatite beads. Those authors suggested that specific bacterial surface structures and adhesins were responsible and that the adhesin was a protease, since its binding could be inhibited by NEM. This is consistent with the 94-kDa protein being the collagenase as well as the adhesin with which the bacteria attach to the collagenous substrata in the periodontum. Alkylation of the thiol groups in the immediate vicinity of the active site of the protease by, for example, NEM would not only lead to protease inactivation but would also prevent binding to the substrate. Attempts to show that the enzyme was surface located by using antibodies raised against SDS-PAGE-purified protease or by electron microscopy binding studies using collagen-coated gold particles proved unsuccessful. Current work is attempting to answer the question of location by generating collagenase-deficient mutants and determining changes in their binding patterns to collagenous substrata.

In conclusion, the 94-kDa collagenase is a protease involved in the cleavage of native triple helical collagen, which may also function as the surface-located adhesin permitting the bacteria to attach to collagenous material. Experiments are now under way to isolate the gene for this enzyme, after which collagenase-negative *P. gingivalis* mutants can be generated by using a novel *Escherichia coli-Bacteroides* shuttle vector system (7b), thus allowing the exact role that this bacterial protease plays in periodontitis to be examined.

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