A Study of the Collagen-binding Domain of a 116-kDa Clostridium histolyticum Collagenase*

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The Clostridium histolyticum 116-kDa collagenase consists of four segments, S1, S2a, S2b, and S3. A 98-kDa gelatinase, which can degrade denatured but not native collagen, lacks the C-terminal fragment containing a part of S2b and S3. In this paper we have investigated the function of the C-terminal segments using recombinant proteins. Full-length collagenase degraded both native type I collagen and a synthetic substrate, Pz-peptide, while an 88-kDa protein containing only S1 and S2a (S1S2a) degraded only Pz-peptide. Unlike the full-length enzyme, S1S2a did not bind to insoluble type I collagen. To determine the molecular determinant of collagen binding activity, various C-terminal regions were fused to the C terminus of glutathione S-transferase. S3 as well as S2bS3 conferred collagen binding. However, a glutathione S-transferase fusion protein with a region shorter than S3 exhibited reduced collagen binding activity. S3 liberated from the fusion protein also showed collagen binding activity, but not S2aS2b or S2b. S1 had 100% of the Pz-peptidase activity but only 5% of the collagenolytic activity of the full-length collagenase. These results indicate that S1 and S3 are the catalytic and binding domains, respectively, and that S2a and S2b form an interdomain structure.

Collagens are the major protein constituents of the extracellular matrix and the most abundant proteins in all higher organisms (1). The tightly coiled triple helical collagen molecule assembles into water-insoluble fibers or sheets which are cleaved only by collagenases, and are resistant to other proteinases. Various types of collagenases, which differ in substrate specificity and molecular structure, have been identified and characterized. Bacterial collagenases differ from vertebrate collagenases in that they exhibit broader substrate specificity (2, 3). Clostridium histolyticum collagenase is the best studied bacterial collagenase (4) and is widely used as a tissue-dispersing enzyme (5, 6). This enzyme is unique in that it can degrade both water-insoluble native collagens and water-soluble denatured ones, can attack almost all collagen types, and can make multiple cleavages within triple helical regions (4). Kinetic studies of collagenases have provided insight into the high-ordered structure of collagens (7, 8). However, the structure-function relationship of this unique enzyme is not known.

Multiple forms of collagenase are produced by C. histolyticum. Seven different forms have been identified, and they are divided into two classes based on possible similarities in their amino acid sequences and their specificities toward peptide substrates (9). In a previous study (10) we have cloned and sequenced a colH gene encoding the 116-kDa collagenase (ColH), which is abundant in many commercial enzymes (11). Comparison of the predicted amino acid sequence of ColH with those of the Vibrio alginolyticus (12) and Clostridium perfringens collagenases (13) revealed a segmental structure for these enzymes (10). ColH has been shown to consist of four segments, S1, S2a, S2b, and S3, and S2a and S2b are homologous. The molecular masses of S1, S2a, S2b, and S3 are 78.1, 10.0, 9.9, and 14.1 kDa, respectively (10). S1 contains the sequence HEXXH, a consensus motif located at the catalytic center of zinc-metalloproteases. A 98-kDa gelatinase that copurified with ColH hydrolyzed denatured but not native collagen. The two enzymes possessed identical N-terminal sequences and their peptide maps were almost identical. Therefore, the 98-kDa gelatinase is probably produced by cleaving off a C-terminal peptide from ColH (10). These observations led us to suspect that the C-terminal peptide forms a functional domain, which is involved in either providing accessibility to or binding of the enzyme to collagen.

To gain insights into the structure-function relationships of C. histolyticum collagenases, we have attempted a molecular dissection of ColH by constructing recombinant derivatives of the enzyme. In this paper, we examined the collagen binding activities of various C-terminal peptides fused to the C terminus of glutathione S-transferase (GST)1 to localize the collagen-binding domain. We have also examined the enzymatic activities of C-terminal truncated species toward native type I collagen and a synthetic peptide substrate.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Enzymes—C. histolyticum JCM 1403 (ATCC 19401) was obtained from the Institute of Physical and Chemical Research (Saitama, Japan). Bacillus subtilis DB104 (14) was used to produce the recombinant collagenases. Escherichia coli DH5a (15) was used for the construction of all recombinant plasmids. Plasmid pCHC116ΔS was generated by nested deletion of pCHC116 (10) from its 3′ end. The plasmid contains a colH gene fragment from nucleotide 2,719 to 3,391 (numbered as in Ref. 10), which encodes the C-terminal segments of ColH, S2b, and S3. E. coli BL21 and the pGEX-4T series plasmids (Pharmacia Biotech, Uppsala, Sweden) were used as a host-vector system for the production of GST fusion proteins. A ColH fraction containing the gelatinase, prepared from C. histolyticum cultures as described previously (10), was used as partially purified ColH. ColH

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1 The abbreviations used are: GST, glutathione S-transferase; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; CB buffer, collagen binding buffer; FP, fusion protein; bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)-propane-1,3-diol.
purified from cultures of recombinant C. histolyticum by column chromatography.

**Growth Conditions**—Recombinant C. histolyticum strains were grown as described previously (16). For screening recombinant plasmids, E. coli DH5α transformants were grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) and 0.5% yeast extract. For the preparation of recombinant proteins, all recombinant E. coli BL21 cells were grown in 2YT-G medium consisting of the following ingredients: 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, 20 g of glucose, and 100 mg of ampicillin/liter. The expression of C. histolyticum was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Wako Pure Chemical Industries, Osaka, Japan).

**Purification of the Recombinant ColH**—An N-terminal peptide of ColH, consisting of S1 and S2a (S1S2a), was constructed as follows. A 2.9-kilobase HindIII-PstI fragment containing the ColH gene (10) was ligated into the EcoRV and PstI sites of Bluescript II KS(+) (Stratagene). The downstream SacI site of the resulting plasmid (pCH200) was used as a synthetic oligonucleotide, 5'-GCTTATATTAAGCAATGCT-3', which was inserted so that translation from a ColH transcript terminates at the TAA codon. The resulting plasmid was designated as pCH201. A 3.1-kilobase BssHII fragment, which encodes amino acids 1 to 766, (numbered as in Ref. 10) corresponding to S1S2a plus an extra 5-amino acid stretch (AvaI-Gly-Ile-His), was isolated from pCH201 and then ligated into the Smal site of pHYS00PKL. The resulting plasmid was transformed into BL21 competent cells and selected as described elsewhere (16). The fractions with Pz-peptidase activity after gel filtration were applied to a hydrophobic interaction chromatography system described elsewhere (16). The fractions with Pz-peptidase activity were eluted by a linear gradient from 40 to 0% ammonium sulfate in 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The fractions with Pz-peptidase activity were eluted by a 280-ml linear gradient from 40 to 0% ammonium sulfate in 50 mM Tris-HCl (pH 7.5). The Pz-peptidase activity eluted at 29% ammonium sulfate. The active fraction was dialyzed against 50 mM Tris-HCl (pH 8.5) and then applied to a MonoQ column. Chromatography was performed as described elsewhere (16) except that the pH was changed to 8.5.

A recombinant N-terminal peptide containing only S1 was produced as follows. An oligonucleotide encoding the C-terminal peptide of S1 (Val94→Ser98) followed by a stop codon was prepared by polymerase chain reaction using two primers (5'-CGGTGGTGGAGGCCTCAACCAAGTC-3' and 5'-CAGCGTTAGAATCCCTCCTTGTA-3') and pCH201 plasmid DNA. The amplified fragment was cloned into the pT7Blue T-vector (Novagen). The fragment was inserted into the T-vector (Novagen) as described elsewhere (16).

**DNA Manipulations and Sequencing**—Restriction endonucleases were purchased from Takara Shuzo Co. (Kyoto, Japan), Toyobo (Osaka, Japan), and New England Biolabs (Beverly, MA). The DNA ligation kit was a product of Takara Shuzo. All recombinant DNA procedures were carried out as described by Sambrook et al. (17). All constructs were sequenced to confirm the reading frame on an automated nucleotide sequencer (model ABI PRISM 377, Perkin-Elmer, Foster City, CA). An ABI PRISM dye terminator cycle sequencing ready kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer), and pGEX primers (Pharmacia) were used for sequencing the GST fusion constructs. A Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Japan Inc., Tokyo, Japan) and M13 dye primes (Perkin-Elmer) were used for sequencing all other constructs.

**Enzyme Assay, Protein Determination, and SDS-PAGE**—The activities of the recombinant collagenases were determined using Pz-peptide (4-phenylazobenzyloxycarbonyl-Pro-Leu-Glu-Pro-Arg, Sigma) or insoluble collagen from bovine Achilles tendon (Worthington Biochemical Co., Freehold, NJ), as described elsewhere (16). The GST activity of GST fusion proteins was assayed using 1-chloro-2,4-dinitrobenzene as described in the supplier’s protocol. Protein concentrations were determined by the Bradford method (19) using the Bio-Rad protein assay reagent (Bio-Rad) with bovine serum albumin (BSA) as a standard. All the assays were carried out in triplicate. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7.5, 12.5, or 15% polyacrylamide gels, and the gels were stained with Coomassie Brilliant Blue R as described previously (20). Band intensity was determined using a flat bed scanner and the public domain computer program, NIH Image (developed at the National Institute of Health, Bethesda, MD, and available from the Internet by anonymous FTP from zippy.nimh.nih.gov).

**Determination of the N-terminal Amino Acid Sequence of C-terminal Fragments**—Two hundred picomoles of the isolated C-terminal fragments obtained from fusion proteins (FP302, FP305, FP342, and FP344) were blotted on a polyvinilidene difluoride membrane using ProSorb devices (Perkin-Elmer) as described by the supplier. Twenty amino acid residues from the N terminus were determined for each fragment on an automatic protein sequencer (Model 492, Perkin-Elmer). All the isolated fragments possessed the expected N-terminal amino acid sequences.

**Determination of the Molecular Mass of a C-terminal Peptide**—A GST fusion protein containing 288S3 (FP302, 1.1 mg) was cleaved by incubation with 10 units of thrombin for 5 h at room temperature. The reaction mixture was dialyzed twice against 2 liters of phosphate-buffered saline (PBS) at 4 °C to remove glutathione, and the cleaved N-terminal peptide was removed by addition of glutathione-Sepharose beads (100 μl). After incubation at room temperature for 30 min, the suspension was centrifuged at 500 × g for 5 min and the bead treatment was repeated three times. The supernatant was dialyzed against 2 liters of distilled water at 4 °C with two changes, filtrated through an 0.45-μm filter, and lyophilized. The sample was dissolved in 100 μl of distilled water, mixed with 3.5-dimethoxy-4-hydroxycinnamic acid solution (10 mg/ml of 50% ethanol), and analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (model Kompact MALDI III, Kratos, Manchester, United Kingdom) with BSA as an internal standard.

**Collagen Binding Assay**—Collagen binding assay was as follows, unless otherwise stated. Five milligrams of insoluble collagen (type I, C-9878, Sigma) were added to an Ultrafree microcentrifugal device with an 0.22-μm low-binding Durapore membrane (Millipore, Bedford, MA), which was placed in a microcentrifuge tube. All steps were carried out at room temperature. Two hundred microliters of collagen binding buffer (CB buffer: 50 mM Tris-HCl, 5 mM CaCl2, pH 7.5) were added to swell the collagen fibers. After incubation for 30 min, the tube was centrifuged at 15,000 × g for 15 min. Centrifugation was repeated after changing the direction of the tube in the rotor. The supernatant was resuspended in 60 μl of CB buffer containing 100 pmol of enzyme and incubated for 30 min. The filtrate was collected by centrifugation at 15,000 × g for 15 min, and used for GST assay or analysis by SDS-PAGE. To determine the binding affinities of ColH(E416D) and various C-terminal segments by Scatchard plot analysis, the collagen binding assay was carried out with the following modification. Collagen was

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pCH116Δ3 (see above) encoding S2b plus S3 (S2bS3) was deleted from its 5’ end using appropriate restriction enzymes. Each fragment was inserted into the Smal site of a suitable GST fusion vector of the pGEX-4T series (Pharmacia) so that the reading frame is intact. After the nucleotide sequence of each fusion gene was confirmed, fusion protein containing GST fragments was expressed by E. coli (BL21). All constructs were sequenced to confirm the reading frame on an automated nucleotide sequencer (model ABI PRISM 377, Perkin-Elmer, Foster City, CA). An ABI PRISM dye terminator cycle sequencing ready kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer), and pGEX primers (Pharmacia) were used for sequencing the GST fusion constructs. A Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Japan Inc., Tokyo, Japan) and M13 dye primes (Perkin-Elmer) were used for sequencing all other constructs.

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mass markers are shown in lane 6. Numbers on the right are molecular masses (in kDa) of the markers. Positions of 116-kDa CoH and 98-kDa gelatinase are indicated by C or G, respectively, on the left.

Collagenase binding of GST-S2bS3 fusion protein and its cleaved product. Molecular mass markers are shown in lane 1. Six micrograms of each protein were incubated at room temperature in the absence (lane 2) or presence (lane 3) of 5 mg of insoluble collagen. The filtrates were subjected to SDS-PAGE on a 12.5% polyacrylamide gel. Molecular mass markers are shown in lane 5. Numbers on the right are molecular masses (in kDa) of the markers.

RESULTS

Binding of CoH and Gelatinase to Collagen—To examine collagen binding, 6 μg of CoH in 60 μl of CB buffer was added to 5 mg of swollen collagen. After the mixture was incubated at room temperature for 30 min and centrifuged, a 20-μl sample of the supernatant was analyzed by SDS-PAGE. As shown in Fig. 1, the gelatinase but not CoH was detectable in the filtrate, suggesting that CoH but not the gelatinase binds to insoluble type I collagen. Various smaller polypeptides were also detected in the filtrate. These might have resulted from hydrolysis of insoluble collagen by CoH. To inhibit the collagenolytic activity of CoH, the incubation was carried out at 4 °C for 30 min, and then the filtrate was analyzed by SDS-PAGE (Fig. 1). The gelatinase was again detectable but neither CoH nor the smaller polypeptides were detected in the filtrate, indicating that CoH binds collagen without degrading it at 4 °C. Therefore, subsequent collagen binding assays of active collagenases were carried out at 4 °C.

The difference in the collagen-binding capability of the two enzymes suggests that the C-terminal peptide which is absent in the gelatinase is involved in collagen binding. However, it has not yet been proved that the 98-kDa gelatinase is produced from CoH by cleaving off the C-terminal region. To test this possibility, two recombinant enzymes, 116-kDa rCoH and its truncated form consisting of S1S2a were purified from cultures of B. subtilis cells. Twenty-five picomoles of each enzyme were mixed with 5 mg of swollen insoluble collagen, and the mixture was incubated at 4 °C for 30 min. A sample containing 1 μg of protein was analyzed by SDS-PAGE (Fig. 2).

While the full-length rCoH bound to insoluble collagen, the truncated form did not. Activities against insoluble collagen and Pz-peptide, a synthetic water-soluble substrate, were determined for the two recombinant polypeptides (Table I). The Pz-peptide hydrolyzing activity of the truncated form was 73% of that of rCoH, while its collagenolytic activity was only 7%. This result suggests that the C-terminal peptide is required for binding and hydrolysis of insoluble collagen.

Collagen Binding of GST Fused to the C-terminal Peptide—The C-terminal peptide, S2bS3, was fused to the C terminus of GST to see if it conferred the ability to bind to collagen on GST. The apparent molecular mass of the fusion protein estimated by SDS-PAGE (Fig. 3), 51 kDa, agreed with the 50,526 Da value calculated from the nucleotide sequence of the corresponding gene. The fusion protein was cleaved by thrombin and SDS-PAGE showed two peptides. One migrated to the same position as the product generated by cleavage of GST alone (26 kDa), and the other (28 kDa) reacted with anti-collagenase antiserum (data not shown). The peptide was purified, its N-terminal amino acid sequence was determined, and it coincided with the predicted sequence. Its apparent molecular mass differed from the value (24,378 Da) calculated for the S2bS3 containing peptide. To determine the molecular mass more accurately, the peptide was analyzed by mass spectrometry. Its molecular mass was determined to be 24,370 Da and the reason for its abnormal mobility in SDS-PAGE is unknown.

### Table I

| Enzyme       | Collagenase | Pz-peptidase |
|--------------|-------------|--------------|
| units/μmol   |             |              |
| rCoH         | 0.164 ± 0.006 | 0.0859 ± 0.0036 |
| rCoH(S1S2a)  | 0.0116 ± 0.0059 | 0.0629 ± 0.0048 |
| rCoH(S1)     | 0.00857 ± 0.00154 | 0.125 ± 0.006 |

FIG. 1. Collagen binding of CoH and gelatinase. Insoluble type I collagen was incubated in CB buffer (lane 1). Six micrograms of partially purified CoH were incubated at room temperature for 30 min in the absence (lane 2) or presence (lane 3) of 5 mg of insoluble collagen. The enzyme was also incubated at 4 °C in the absence (lane 4) or presence (lane 5) of insoluble collagen. The filtrates were subjected to SDS-PAGE on a 7.5% polyacrylamide gel. Molecular mass markers (lane 6). Numbers on the right are molecular masses (in kDa) of the markers. Positions of 116-kDa CoH and 98-kDa gelatinase are indicated by C or G, respectively, on the left.

FIG. 2. Collagen binding of rCoH and its truncate form. Twenty-five picomoles of rCoH were incubated at 4 °C in the absence (lane 1) or presence (lane 2) of 5 mg of insoluble collagen. Twenty-five picomoles of the rCoH truncate consisting of S1S2a was incubated at 4 °C in the absence (lane 3) or presence (lane 4) of insoluble collagen. The filtrates were subjected to SDS-PAGE on a 7.5% polyacrylamide gel. Molecular mass markers are shown in lane 5. Numbers on the right are molecular masses (in kDa) of the markers.

FIG. 3. Collagen binding of the GST-S2bS3 fusion protein and its cleaved product. Molecular mass markers are shown in lane 1. Six micrograms of each protein were incubated at room temperature in the absence (lane 2) or presence (lane 3) of 5 mg of insoluble collagen. The filtrates were subjected to SDS-PAGE on a 12.5% polyacrylamide gel. GST-S2bS3, the C-terminal peptide S2bS3 fused to the C terminus of GST; GST, GST moiety generated by thrombin cleavage; S2bS3, the C-terminal peptide S2bS3 moiety generated by thrombin cleavage. Numbers on the left are molecular masses (in kDa) of the markers.

The values represent the average of triplicate trials with the deviations.
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FIG. 4. Graphic representation and collagen binding of peptide S2bS3 deletions. A, five GST fusion proteins, FP302 to FP306, were constructed by using DNA fragments of pCHIC116A3 digested with appropriate restriction enzymes and a suitable GST fusion vector of the pGEX-4T series. They were purified by affinity chromatography on a glutathione-Sepharose column. B, 2 μg of each GST fusion protein were analyzed by SDS-PAGE on a 12.5% polyacrylamide gel. Lane 1, markers; lane 2, FP302; lane 3, FP303; lane 4, FP304; lane 5, FP305; lane 6, FP306; lane 7, GST. Numbers on the left are molecular masses (in kDa) of the markers. C, each fusion protein was cleaved by thrombin, and then 100 pmol of the cleaved product were incubated with (+) and without (−) 5 mg of insoluble collagen. An aliquot (2 μg of protein) of the filtrate was analyzed by SDS-PAGE on a 15% polyacrylamide gel. Numbers on the left are molecular masses (in kDa) of the markers.

Samples of the GST fusion protein before and after cleavage with thrombin were combined, added to CB buffer containing BSA, chicken ovalbumin, and horse myoglobin (6 μg each) and then the final volume was adjusted with CB buffer to 60 μl. After incubation with 5 mg of insoluble collagen, and centrifugation, the supernatant was analyzed by SDS-PAGE (Fig. 3). All proteins except the fusion protein and S2bS3 were present in the filtrate, indicating that S2bS3 and the GST fusion protein specifically bound to collagen.

To determine the collagen binding condition, the S2bS3 peptide was incubated with insoluble collagen under various conditions. When 20 μg of the peptide was incubated with varying amounts of insoluble collagen for 30 min at room temperature, it was almost completely bound to 10 mg or more collagen. When 20 μg of the peptide was incubated with 15 mg of collagen for varying times, approximately 70% of the peptide bound to collagen after 2 min, and the binding reached its maximal level after 30 min. It was shown that the binding capacity of collagen for the S2bS3 peptide is more than 2 μg/mg collagen, and that incubation at room temperature for 30 min is sufficient for completion of the binding reaction.

The effect of pH on collagen binding was examined by determining the GST activity of the GST fusion protein in the filtrate after incubating using the standard assay except the buffer was changed to: 50 mM bis-Tris-HCl containing 5 mM CaCl₂ (pH 5.0) or CB buffer (pH 7.0) or CB buffer (pH 9.0). Mean ± S.D. of collagen binding at pH 5, 7, and 9 were 97.9 ± 0.0, 97.5 ± 0.3, and 100 ± 0.0%, respectively. The Pz-peptidase activity of rColH determined in these buffers was 0.98 ± 0.98, 76.5 ± 2.4, and 60.1 ± 1.2 units/nmol, respectively. When 5 mM EDTA was added to the buffer in place of 5 mM CaCl₂, the Pz-peptidase activity of rColH was completely inhibited, while the collagen binding activity of the GST fusion was 88.6 ± 1.2%. The addition of gelatin at a final concentration of 10 mg/ml to CB buffer did not affect binding of the GST fusion protein (96.0 ± 0.2%). The addition of sodium chloride at a final concentration of 1.5 m slightly affected binding (92.3 ± 0.3%).

Deletion Analysis of the Collagen-binding Domain—Two truncated peptides, S1S2a and S2bS3, exhibited Pz-peptidase and collagen binding activities, respectively. Given the homology between S2a and S2b, it appears that S3 is a collagen-binding domain, and that S2b and possibly S2a form an inter-domain structure. To examine this possibility, S2bS3 was deleted to various lengths from its N terminus and fused to the C terminus of GST (Fig. 4A). The fusion proteins were purified to homogeneity as shown in Fig. 4B. These fusion proteins were incubated with insoluble collagen, and the GST activities in the filtrates were determined (Table II). Since GST activity differed slightly from one fusion protein to another, the collagen binding activity of each fusion protein was determined by calculating the difference of the GST activity in supernatants incubated with and without collagen. As the C-terminal peptide was shortened, collagen binding activity decreased. However, the binding activity of the fusion protein containing S3 (FP305) was still high, being more than 90% of that containing S2bS3 (FP302). When the C-terminal peptide was shorter than S3, binding decreased significantly. The fusion proteins were cleaved by thrombin, and the ability of the C-terminal peptides to bind insoluble collagen were tested by SDS-PAGE (Fig. 4C). S3 alone bound collagen and the shorter peptide bound weakly, as was expected. The difference in band intensity between peptides incubated with and without collagen matched the binding activity of the corresponding fusion protein determined by its GST activity.

Scatchard Analysis of Collagen Binding—Since GST is known to exist as a dimer (21), GST fusion proteins may also exist as a dimer. Therefore, the GST fusion proteins are not suitable for the quantitative evaluation of the collagen binding affinity of various C-terminal segments. Thus, the fusion proteins were cleaved by thrombin, and the collagen binding assay was performed with varying concentrations of the purified C-terminal peptides. Scatchard analysis of the data (Fig. 5) showed that S3 alone bound to insoluble collagen with low but
The site of the collagen-binding domain was examined using GST fusion proteins with varying sized C-terminal fragments. All of the fusion proteins (FP302 to FP306) bound efficiently to gluthathione affinity columns. Since binding requires normal function of the GST moiety, it seems that these C-terminal fragments did not affect significantly the conformation of GST. Qualitative analysis by SDS-PAGE showed that S3 isolated from FP305 bound well to insoluble collagen, and that further deletion caused a marked decrease in its binding activity (the peptide from FP306). Scatchard analysis confirmed that S3 had collagen binding activity, although its $K_d$ value was 2 orders of magnitude higher than that of the mutant full-length enzyme. Therefore, S3 can be considered as a minimal region required to form a collagen-binding domain. The S3 in the construct, FP305, starts at Pro$^{661}$. Since proline residues are often at the termini of α-helices or β-strands, the residue may be located at the beginning of the domain structure. Purification of GST fusion proteins containing shorter fragments such as one from Thr$^{224}$ to Arg$^{681}$ and one from Pro$^{654}$ to Arg$^{681}$ by affinity chromatography was unsuccessful probably due to interference with binding to gluthathione by these shorter fragments. Therefore, it seems likely that the first half of S3 is essential for the formation of the domain structure. In C. perfringens collagenase, CoLA, the S3 homologue is tandemly repeated at the C terminus. Whether or not this repeat enhances collagen binding activity remains to be determined.

There is no significant similarity in amino acid sequence between bacterial and eukaryotic collagenases. The three-dimensional structure of a fibroblast collagenase determined by Li et al. (25), has two domains, a catalytic domain (161 amino acid) and a hemopexin-like one (189 amino acid), which are connected by a short linker sequence (17 amino acid). The latter domain, which is believed to participate in the recognition of the macromolecular substrate, has a four-bladed β-propeller structure consisting of 16 β-strands. The secondary structure of S3 (∼120 amino acid) predicted by the method of
Chou and Fasman (26) and Garnier et al. (27) contains at most 9 β-strands. Considering the necessity of at least six blades for the formation of a stacked propeller structure suggested by Murzin (28) and the four-bladed perpendicular propeller structure of the eukaryotic collagen-binding domain, it seems unlikely that S3 forms a β-propeller structure. Determination of the three-dimensional structure of the collagen-binding domain in bacterial collagenases would facilitate understanding of the molecular mechanism for the interaction between these enzymes and collagen.

Although neither S2aS2b nor S2b showed any binding activities, addition of S2b (FP302) to S3 increased its binding affinity close to that of the full-length enzyme. This enhancement could be explained by the hypothesis that S2b assists proper folding of the minimal collagen-binding domain, S3. There is an alternative explanation that S3 bound to insoluble collagen could modify the local structure of the substrate, where S2b could bind subsequently. The International Polycystic Kidney Disease Consortium (29) showed that the amino acid sequence of S2 is homologous to that of a repetitive sequence (PKD domain) of the protein encoded by the PKD1 gene, the most common gene responsible for autosomal dominant polycystic kidney disease. They speculated that the S2-encoding fragment has been horizontally transferred from eukaryotic cells to prokaryotic cells. It may be possible that the bacterial collagenase genes have evolved from an ancestral bacterial zinc-metalloprotease gene by insertion and duplication of a short stretch between the catalytic domain and the binding domain. Further precise characterization of S2 is necessary to elucidate its functional role.

The collagen binding activity of the C-terminal segments was not inhibited by gelatin, a denatured collagen which lacks a triple helical structure, suggesting that the interaction of the collagen-binding domain with native collagen is conformation-dependent. Type I insoluble collagen exhibits a maximal binding capacity for rColH(E416D) of 108 pmol of enzyme/mg of collagen, which gives an average of one binding site every 32.5 tropocollagen molecules. It is likely that only binding sites on the surface of the collagen fibrils are accessible to the collagenase. Binding of the enzyme may occur only at the sites where multiple tropocollagen molecules form a staggered array of collagen fibrils, where there is a hole between tropocollagen molecules. The C-terminal peptides are smaller and hence could access more binding sites than the full-length enzyme. All of the C. histolyticum collagenases initially cleave collagen molecules at hyperactive sites, and thereafter successively degrade the fragments into small, diacylpeptide peptides (7). Hyperactivity might result from the selective binding of the enzyme to specific sites.

Preliminary experiments showed that a fusion protein carrying the C-terminal fragment (S2bS3), when injected subcutaneously into nude mice, remained at the sites of injection for up to 10 days. Consequently, the collagen binding ability of the C-terminal fragment is not restricted to the substrate preparation used in the present study but it can bind to “living collagen fibers” in vivo, suggesting an important role of the segments in the tissue degrading activity of ColH.

All C. histolyticum collagenases contain one zinc molecule (4), and its depletion by treatment with 1,10-phenanthroline abolishes their hydrolytic activity. Within S1 there is a zinc binding consensus sequence, HEXXH, which is at the catalytic center in other zinc-metalloproteases. Since a truncated enzyme, rColH(S1), showed higher activity against Pz-peptide than the full-length enzyme, the catalytic center seems to be folded into its normal conformation without S2a, S2b, or S3. Further deletions abolished its catalytic activity, and, thus, S1 seems to be the minimal region necessary to form a catalytically active structure. The residual collagenolytic activity of the truncated enzymes may reflect catalytic activity dependent on random collision which is greatly stimulated by a collagen-binding domain. Alternatively, swollen type I collagen may be partly denatured and the residual activity may be due to the gelatinolytic activity of the peptide. Since a collagenase assay using an excess amount of the truncated enzyme reached a saturation level, the latter case seems to be likely. More precise kinetic study is necessary to draw a final conclusion about this point.

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A Study of the Collagen-binding Domain of a 116-kDa \textit{Clostridium histolyticum} Collagenase
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