Human skin collagenase is secreted by cultured fibroblasts in a proenzyme form and can be activated to a catalytically competent enzyme by a number of processes. All modes of activation studied lead to conversion of the proenzyme to a stable 42-kDa active enzyme, concomitant with removal of an 81-amino acid peptide from the amino-terminal end of the molecule. The sequence of events leading to the formation of this enzyme form has been determined by analyzing the primary structure of the conversion intermediates. Trypsin-induced activation of procollagenase occurs as a result of the initial cleavage of the peptide bond between Arg-55 and Asn-56, generating a major intermediate of 46 kDa. Treatment of the proenzyme with organomercurials, which have no intrinsic ability to cleave peptide bonds, initially results in activation of the enzyme without loss of molecular weight. This is followed by conversion to two lower molecular weight species of 44 and 42 kDa, the latter corresponding to the stable active enzyme form. The final cleavage producing this form of collagenase is not restricted to a single polypeptide bond but can occur on the amino-terminal side of any one of three contiguous hydrophobic residues, Phe-100, Val-101, Leu-102. The data suggest that both trypsin and organomercurials activate procollagenase by initiating an intramolecular autoproteolytic reaction resulting in the formation of a stable 42-kDa active enzyme species.

The degradation of interstitial collagen in mammalian species is initiated by specific metalloendoproteases, collagenases (EC 3.4.24.7), which are secreted and found in the extracellular matrix in a zymogen form. Recently, the complete primary structure of a cDNA clone representing the full-size human skin collagenase mRNA has been presented (1). This collagenase is synthesized as a preproenzyme, M, 54,092, and secreted as two forms, a major M, 52,000 proenzyme and a minor glycosylated species, M, 57,000 (2). The activation of procollagenases from a variety of tissues has been reported to occur by several mechanisms. These include proteolysis by trypsin (3, 4, 7, 8), incubation with chaotropic ions (9, 10), the action of nonenzymatic changes and proteolytic cleavage occur during procollagenase activation. This study investigates the nature of the proteolytic events leading to the conversion of procollagenase to the active enzyme by identifying the primary structure of the major intermediates produced during zymogen activation with trypsin or organomercurials.

MATERIALS AND METHODS

Procollagenase was purified from human skin fibroblast conditioned medium by using ammonium sulfate precipitation, carboxymethylcellulose chromatography, and gel filtration on Ultrogel AcA-44 (LKB, Inc.), as previously described (14). The activation of procollagenase by trypsin—Procollagenase at concentrations between 45 and 140 μg/ml in 0.05 M Tris-HCl, pH 7.5, and 0.01 M CaCl₂ was incubated with TPCK-treated trypsin at a 1:50 (w/w) trypsin to procollagenase ratio for 15 min at 0 °C. Under these conditions of proteolysis a major intermediate conversion product was obtained. Subsequent incubation of this reaction mixture at 37 °C resulted in the complete conversion of the proenzyme into the stable active enzyme form. To achieve complete activation, with no accumulation of intermediate forms, procollagenase was incubated in the same buffer with trypsin at a 1:2 (w/w) trypsin to procollagenase ratio at 25 °C for 15 min. The reaction was terminated by the addition of phenylmethylsulfonyl fluoride to a final concentration of 1.0 mM and/or by a 10-fold molar excess of soybean trypsin inhibitor. Samples were dialyzed versus 0.1 M acetic acid, concentrated by lyophilization, and analyzed by SDS-polyacrylamide gel electrophoresis. All samples were analyzed versus 0.1 M acetic acid, concentrated by lyophilization, and analyzed by SDS-polyacrylamide gel electrophoresis.

The abbreviations used are: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SSS, sodium dodecyl sulfate; PMC, phenylmercuric chloride; pHMB, p-hydroxymercuribenzoate; PAGE, polyacrylamide gel electrophoresis.
were run on 10% gels and stained with Coomassie Blue for direct visualization. Gels for electrophoresis were not stained prior to transfer. Collagenase activity was determined by assaying aliquots of the reaction mixture for 45 min using reconstituted [14C]glycine-labeled guinea pig skin collagen fibrils (50,000 cpn/mg of collagen) as a substrate gel as described previously (7).

**Activation of Procollagenase by Organomercurials—**Stock solutions of 0.01 M PMC and 0.01 M pHMB in 0.1 N NaOH were prepared just prior to use. Proenzyme samples were adjusted to 0.05 M Tris-HCl, pH 7.5, to avoid significant changes in pH upon addition of the organomercurial compound. Procollagenase was incubated with a final PMC concentration of 0.5 mM for varying times (0–16 h) at 37°C or with a final pHMB concentration of 0.5 mM for 10 and 60 min at 37°C. Samples were dialyzed versus 0.1 M acetic acid, concentrated by lyophilization, and analyzed by SDS-polyacrylamide gel electrophoresis. Collagenase activity was determined by a 45-min assay (see above) on aliquots of the sample prior to dialysis.

**Electrophoresis and Sequencing—**Protein sequencing was performed on an Applied Biosystems 470A gas phase Sequencer. Samples were electrophoresed onto electroblot onto activated glass fiber paper from SDS-polyacrylamide gels and loaded directly onto the Sequencer by the method of Aebersold et al. (15). Identification of the phenylthiobutyldiainit derivatives was performed by high pressure liquid chromatography as previously described (16).

**RESULTS**

Human fibroblast procollagenase is secreted as a set of twozymogens with molecular weights of 57,000 and 52,000 (14). Recent studies have demonstrated that the two forms are composed of the same polypeptide chain (1) and that the M, 57,000 proenzyme species represents a glycosylated form of the M, 52,000 procollagenase polypeptide (2). Since it is possible to chromatographically separate the two forms to greater than 95% homogeneity and both forms appear to activate and convert in a parallel manner, the investigations presented here have been conducted primarily with the M, 52,000 nonglycosylated polypeptide chain.

**Mechanism of Procollagenase Activation by Trypsin—**When the 52-kDa nonglycosylated human fibroblast collagenase was treated with TPCK-trypsin, two separate conversion products were electrophoretically distinguished at levels sufficient for electrophoresing and sequencing. Complete conversion of procollagenase to the stable 42-kDa active species (Fig. 1, lane 2) can be achieved by treatment with 1:2 (w/w) trypsin to procollagenase ratio at 25°C. Conversion occurs rapidly and, although transient intermediates can sometimes be seen, they are difficult to isolate in sufficient amounts for sequencing. It should be noted that prior to conversion, both the 57- and 52-kDa procollagenase molecules have blocked amino termini. The amino-terminal sequence of the 42-kDa active enzyme form produced by trypsin is Val-Leu-Thr-Glu-Gly-Asn (Fig. 2). When the reaction temperature was maintained at 0°C (Fig. 1, lane 3). This intermediate has previously been shown (8) to lack activity toward collagen substrates. The amino-terminal sequence of this species was found to be Asn-Ser-Gly-Pro-Val-Val-Glu and corresponds to positions 56–62 of the procollagenase sequence (Fig. 2). If the reaction temperature was subsequently raised to 37°C the 46-kDa intermediate converted to a 42-kDa enzyme identical to that generated when the conversion is initiated at 37°C (8). The results presented in Table 1 show that the formation of the 42-kDa active enzyme under these conditions does not require trypsin activity, but can be blocked completely by the addition of EDTA, and prevents the formation of the 46-kDa trypsin-activated enzyme.

**Table 1.** Analysis of the trypsin activation of human procollagenase by SDS-PAGE. Lane 1, untreated procollagenase (52 kDa, Fig. 2A); lane 2, procollagenase activated with TPCK-trypsin at 1:2 (w/w) ratio at 25°C for 15–60 min (42 kDa, Fig. 2E); lane 3, procollagenase treated with TPCK-trypsin at a 1:50 (w/w) ratio at 25°C for 15–60 min (46 kDa, Fig. 2B).

| Description | Species      | Sequence | Position |
|-------------|--------------|----------|----------|
| A) Procollagenase | 52 kDa (51,929) | "Blocked" | (20)     |
| B) Trypsin treated | 1:50 (w/w), 0°C | 46 kDa (47,531) | NSCPFVE | 56        |
| C) PMC treated | 15 min. (upper band) | 44 kDa (44,471) | LKXQGCF | 84        |
| D) PMC treated | 16 h (lower band) | 42 kDa (42,717) | LEKOVMT | 100       |
| E) Trypsin treated | 1:2 (w/w), 25°C | 42 kDa (42,750) | VTOLGQAEK | 101       |

**FIG. 1.** Amino-terminal sequence and location of the trypsin and PMC activation products in human pro-collagenase. The sequence at the bottom of the figure shows only the first 120 residues of the 469-residue pro-collagenase sequence with the initiator methionine of the signal peptide designated residue 1. The size of the fragments as determined from the SDS gels is listed (kDa), and the molecular weights calculated from the amino acid sequence are listed in parentheses. The cleavage of the signal peptide is predicted to occur between residue 19 and 20 (1), and this position was used to calculate the molecular weight of the procollagenase. Time intervals indicate that the majority of the product is generated and remains relatively constant over the period indicated.
Sequence of Procollagenase Activation Products

**TABLE I**

Autocatalysis of the initial trypsin-derived intermediate of procollagenase: concentration dependence and effect of EDTA

| Protein concentration | Initial intermediate remaining at | % |
|-----------------------|----------------------------------|---|
|                      | 0 h                               | 1 h | 3 h |
| 10 µg/ml              | 100                               | 78  | 32 |
| 25 µg/ml              | 100                               | 73  | 36 |
| 50 µg/ml              | 100                               | 65  | 30 |
| 50 + EDTA             | 100                               | 97  | 101 |

is independent of the initial procollagenase concentration in the range of 10–50 µg/ml. The latter observation is in agreement with the results reported by this laboratory previously for enzyme concentrations in the range of 100–800 µg/ml (8).

**Mechanism of Procollagenase Activation by Organomercurial Compounds**—Incubation of procollagenase with organomercurials such as PMC also results in enzyme activation and the conversion to the two major lower molecule weight species shown in Fig. 3. The first conversion product, 44 kDa (Fig. 3, lane 1), is generated rapidly, while conversion to the second (Fig. 3, lane 6), which corresponds to the 42-kDa species described above, is formed after a longer incubation period. The amino-terminal sequence of the 44-kDa species was found to be Leu-Lys-Val-Met-Lys-Gln and corresponds to positions 84–89 in the preprocollagenase sequence (Fig. 2). The 42-kDa species from this conversion does not routinely produce a single sequence but rather displays a pattern corresponding to staggered cleavage around position 101. The residue identifications at each position clearly indicate that the sequences Phe-Leu-Thr-Glu-Gly-Gln, Val-Leu-Thr-Glu-Gly-Gln, and Leu-Thr-Glu-Gly-Gln are all present. Furthermore, the relative ratios of the three sequences differ from experiment to experiment without a particular sequence consistently predominating.

Treatment of procollagenase with the organomercurial pHMB resulted in the rapid appearance of enzymatic activity (20–40% of the total potential activity within 10 min). However, confirming our previous observations (8), conversion to lower molecular weight forms, as had been previously described for PMC, was minimal and did not correlate with the attainment of activity. Even after generation of maximal enzymatic activity (60 min), conversion to lower molecular weight species remained limited. As shown in Fig. 4, the 52-kDa species is the major form present. Only at later times, well after generation of substantial activity (8), do lower molecular weight forms appear which correspond to those seen during PMC activation. Sequence analysis of the major 52-kDa species demonstrated that the amino terminus remained blocked after a 10- or 60-min incubation period. Amounts of the lower molecular weight forms obtained over the 60-min time period were insufficient for sequence analysis.

**DISCUSSION**

The amino-terminal sequences of the major conversion polypeptides produced by two different modes of activation of human fibroblast collagenase have been determined and located in the sequence of the proenzyme. The results indicate that the conversion products are generated by the cleavage of peptides from the amino-terminal portion of the proenzyme. Although removal of peptides from the carboxyl-terminal
portion of the proenzyme cannot be excluded, the close correlation between the calculated molecular weight of the peptides lost upon conversion and the reduction in molecular weight of the main polypeptide chain as determined by SDS-PAGE makes this possibility unlikely.

Treatment of procollagenase with relatively low levels of trypsin produced the largest intermediate conversion product. This enzyme species resulted from cleavage of the polypeptide bond between Arg-55 and Asn-56 which is consistent with the specificity of trypsin. Although other potential trypsin sites are apparent in the sequence of the proenzyme, further digestion does not occur as long as the temperature is maintained at 0°C. Raising the temperature to 37°C in the presence of large quantities of trypsin inhibitors resulted in the production of lower molecular weight conversion products which ultimately are converted to the stable 42-kDa active collagenase. This conversion, which takes place in the presence of phenylmethylsulfonyl fluoride and soybean trypsin inhibitor, is completely inhibited by EDTA, suggesting a metalloenzyme activity. Treatment of the proenzyme with TPCK-trypsin at elevated temperatures (25–37°C) also produced an identical final product with Val-101 at the amino terminus. However, sequence analysis indicates that the peptide bond cleaved to ultimately produce this 42-kDa product is not consistent with the usual specificity of trypsin. The reason for this becomes clear when one considers the results of organomercurial activation.

The activation initiated by organomercurials (PMC and pHMB), which have no intrinsic ability to cleave peptide bonds, also stimulates the proteolytic conversion of the proenzyme to lower molecular weight species ultimately leading to the formation of the 42-kDa active enzyme. The peptide bonds which have been identified as being cleaved during this process are between Thr-83 and Leu-84, Gln-99 and Phe-100, Phe-100 and Val-101, and Val-101 and Leu-102. The nature of these cleaved bonds, one of which is identical to the final cleavage site produced during zymogen conversion in response to trypsin at 25–37°C, is most consistent with a neutral protease type of activity. That is, a specificity dictated primarily by the residue contributing the amino group of the susceptible bond with a general preference for hydrophobic residues such as phenylalanine, valine, leucine, isoleucine, methionine, and tryptophan in that position.

The results indicate that the initial event in treatment of the proenzyme with organomercurials is probably a conformational change leading to the rapid appearance of collagenolytic activity without a concomitant loss of molecular weight. This is supported by the observation that pHMB results in the rapid appearance of enzyme activity even though conversion to the lower molecular weight forms is delayed and the amino terminus of the enzyme remains blocked even when maximal enzyme activity is attained. These observations suggest that the proteolytic cleavage responsible for the formation of the 42-kDa stable enzyme species is an autocatalytic reaction requiring initial activation by organomercurials or trypsin. The complete inhibition of this reaction by EDTA and the nature of the peptide bond cleaved indicates the involvement of a metalloprotease and supports the above interpretation. In addition, this cleavage is likely to be intramolecular since both the rate of conversion by PMC and trypsin into lower molecular weight species is not affected by enzyme concentration or by the addition of purified active enzyme (8).

Taken together these observations suggest that the stable activated 42-kDa enzyme is generated in a two-step reaction. The first step involves the activation of procollagenase induced either by proteolytic cleavage (trypsin) or conformational rearrangement (organomercurial compounds). The second step is an autoproteolytic intramolecular reaction leading to formation of the stable active enzyme.

While all known modes of activation eventually lead to conversion of the 52-kDa procollagenase to the 42-kDa active collagenase, the results of this investigation show that the production of the 42-kDa species does not necessarily occur as a result of the cleavage of a unique peptide bond. Rather, cleavage may occur at any one of three peptide bonds which are susceptible to neutral protease activity in a limited area around residues 92–102. Although it is not known what directs the cleavage to this area, the lack of selectivity for a specific bond suggests that it may be dictated by the tertiary structure of the enzyme. This region of the molecule may represent the most distal residues from the amino terminus that are susceptible to neutral proteases because it is in the vicinity of a transition between an exposed polypeptide backbone and a tightly packed globular region inaccessible to proteolytic attack. In this regard, procollagenase might be envisioned as a globular protein with an extended tail at the amino terminus which, during conversion, gets progressively cleaved until the globular region is encountered and proteolysis can go no further. Similarly, activation in the absence of, or prior to, a proteolytic event can be viewed as a change in the orientation of the tail which exposes a competent catalytic site and renders the tail susceptible to autoproteolysis. This model is analogous to that proposed for the activation of pepsinogen which undergoes a proteolytic event in the absence of exogenous proteases and in response to low pH (17, 18). Although confirmation of this hypothesis will require direct crystallographic data, it does serve to explain the experimental observations.

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REFERENCES
1. Goldberg, G. I., Wilhelm, S. M., Kronberger, A. M., Bauer, E. A., Grunt, G. A., and Eisen, A. Z. (1986) J. Biol. Chem. 261, 6600–6605
2. Wilhelm, S. M., Eisen, A. Z., Tetas, M., Clark, S. D., Kronberger, A., and Goldberg, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3765–3769
3. Vater, G. (1972) Biochem. J. 126, 275–289
4. Harper, J. E., Bloch, K. J., and Gross, J. (1971) Biochemistry 10, 3035–3041
5. Birckelad-Hansen, H., Cobb, C. M., Taylor, R. E., and Pullinger, H. M. (1976) J. Biol. Chem. 251, 3161–3168
6. Wehr, Z., Mainardi, C. L., Vater, C. A., and Harris, E. D., Jr. (1977) N. Engl. J. Med. 296, 1017–1023
7. Bauer, E. A., Stricklin, G. P., Jeffrey, J. J., and Eisen, A. Z. (1975) Biochemistry 14, 2475–2483
8. Stricklin, G. P., Jeffrey, J. J., Roswit, W. T., and Eisen, A. Z. (1983) Biochemistry 22, 61–69
9. Abe, S., and Nagai, Y. (1972) J. Biochem. (Tokyo) 71, 919–922
10. Abe, S., Shinnosuke, M., and Nagai, Y. (1975) J. Biochem. (Tokyo) 73, 1007–1011
11. Tyson, E., Seltzer, J. L., Halme, J., Jeffrey, J. J., and Eisen, A. Z. (1981) Arch. Biochem. Biophys. 208, 440–443
12. Vater, C. A., Nagase, H., and Harris, E. D., Jr. (1985) J. Biol. Chem. 258, 5374–5382
13. Sellers, A., Cartwright, E., Murphy, G., and Reynolds, J. J. (1977) Biochem. J. 163, 263–277
14. Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., and Eisen, A. Z. (1977) Biochemistry 16, 1081–1085
15. Aebi, R. H., Taplow, D. B., Hood, L. E., and Kent, S. B. H. (1986) J. Biol. Chem. 261, 4222–4229
16. Grant, G. A., Sacchettini, J. C., and Welgus, H. G. (1985) Biochemistry 24, 354–358
17. Tang, J. (1976) Trends Biochem. Sci. 1, 205–208
18. James, M. N. G., and Sielecki, A. R. (1986) Nature 319, 33–38

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