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ACTIVATION OF HUMAN LEUKOCYTE COLLAGENASE BY COMPOUNDS REACTING WITH SULFHYDRYL GROUPS *

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Summary

Human collagenase was partially purified from the granules of polymorphonuclear leukocytes by gel filtration, and the effects of two sulfhydryl reagents, *N*-ethylmaleimide and *p*-aminophenylmercuric acetate, on the enzyme activity were studied. The enzyme activity was assayed by incubating with soluble [^{14}C]proline-labeled type I collagen, and the rate of collagen cleavage was quantitated by isolating the specific cleavage products by SDS-polyacrylamide gel electrophoresis. Results demonstrated that the collagenase, which was at first mostly in a latent form, was rapidly activated by these two sulfhydryl reagents. The enzyme activity, however, returned gradually to the control level in the presence of the sulfhydryl reagent or after removal of an excess of the reagent. The enzyme activity, after activation with *N*-ethylmaleimide, could be returned to the control level by the addition of a 2-fold molar excess of cysteine. The heat stability of the enzyme activity before and after activation by *N*-ethylmaleimide was also tested. The results indicated that the initial enzyme activity, before the activation, was stable at 60°C for at least 5 min, and the enzyme could be subsequently activated by *N*-ethylmaleimide to the same extent as an unheated control. If, however, the enzyme was first activated by incubating in the presence of *N*-ethylmaleimide and subsequently incubated at 60°C, a marked decrease in the enzyme activity as a result of the 5 min

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heating was noted. The results of the present study indicate that human leukocyte collagenase can be activated by compounds reacting with thiol groups.

Introduction

Human polymorphonuclear leukocytes have previously been shown to contain a collagenase which is similar to collagenases from various other vertebrate sources (Refs. 2—7; for reviews on collagenases, see Refs. 8—10, 34, 35). It cleaves the native collagen into two fragments, TC^A and TC^B. These designations refer to triplehelical fragments derived from cleavage of a native collagen molecule by mammalian collagenase, representing three-quarters and one-quarter of the length of the original collagen molecule, respectively. Under denaturing conditions, these fragments can be further dissociated into individual polypeptide chains α^A and α^B. Since the initial cleavage of collagen by a collagenase is the committed, rate-limiting step in collagen degradation, it is clear that changes in the enzyme activity play an important role in normal turnover of collagen. In addition, changes in the activity of collagenases may play a role in the pathophysiology of several human diseases, such as rheumatoid arthritis, osteoarthrosis and periodontal disease [11—17]. In these conditions, the participation of human leukocyte collagenase may be of particular importance in the inflammatory stages of the disease processes.

The collagenases isolated from various sources are present mostly in an inactive, latent form, and they can be activated by various means, such as a proteolytic treatment with trypsin [18—22]. Several additional factors, such as serum components, as well as tissue inhibitors and activators have been shown to modify the activities of a number of vertebrate collagenases [23—26]. Of particular interest are the observations that compounds reacting with sulfhydryl groups effectively activate collagenases [27—30]. In the present study we have investigated the effects of two compounds which react with sulfhydryl groups, *N*-ethylmaleimide and *p*-aminophenylmercuric acetate on the activity of human leukocyte collagenase. Recent development of a specific and sensitive assay for collagenase activity, based on the quantitative isolation of specific cleavage products of the collagenase reaction, has allowed us to examine the properties of the leukocyte collagenase in a partially purified form.

Materials and Methods

Partial purification of leukocyte collagenase

For partial purification of collagenase, buffy coats of 6—17 l human blood were obtained from the hematological laboratories of the Finnish Red Cross. The polymorphonuclear leukocytes were isolated by Dextran sedimentation by mixing cell suspensions with an equal volume of 0.9% NaCl and 2.2% Dextran (Pharmacia, T-500). The mixture was incubated for 30 min at 22°C, the upper layer containing the polymorphonuclear leukocytes was collected, and the cells were isolated by centrifugation. The cell pellet was washed three times with 0.9% NaCl and then briefly suspended in distilled water to lyse contaminating red blood cells. The leukocytes were again collected by centrifugation and

washed with 0.34 M sucrose. The cell pellet was then homogenized in 0.34 M sucrose for 2 min with a Teflon and glass homogenizer, and the homogenate was centrifuged at 15 000 × *g* for 20 min [4]. The supernatant (defined as post-granular supernatant) was discarded, and the pellet containing leukocyte granules was washed with 0.05 M Tris-HCl buffer (pH 7.8), containing 0.2 M NaCl and 5 mM $CaCl_2$. The pellet was then suspended in the same buffer and freeze-thawed five times, the suspension was then centrifuged at 30 000 × *g* for 60 min. The supernatant was applied to a 4 × 60 cm column of Sephacryl S-200 (Pharmacia). The column was equilibrated and eluted with 0.2 M NaCl and 5 mM $CaCl_2$ in 0.05 M Tris-HCl (pH 7.5); 6-ml fractions were collected and assayed for collagenase activity. The fractions containing the collagenase activity were pooled and used for subsequent studies. The sulfhydryl reagents were added to the enzyme preparations using freshly prepared solutions and at the end of the incubation periods, the activity of collagenase was measured.

Assay for collagenase activity

The enzyme activity was detected by a method employing soluble, [^{14}C]-proline-labeled chicken type I collagen as a substrate, as described previously by us [6]; the specific radioactivity of the substrate was $2.5 \cdot 10^4$ dpm/mg. The enzyme incubations were performed with 1.65 mg/ml of type I collagen substrate in 0.05 M Tris-HCl buffer (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$, at 25°C as described elsewhere [6]. The specific degradation products resulting from the collagenase cleavage were then isolated by polyacrylamide disc gel electrophoresis in SDS. The rate of collagen cleavage was expressed as cpm ^{14}C radioactivity recovered in the α^A and α^B peptides.

Results

Partial purification of collagenase from human polymorphonuclear leukocytes

For purification of the leukocyte collagenase, whole blood was centrifuged and the buffy coat containing white blood cells was collected. A preparation containing the leukocyte granules was then obtained by homogenization and centrifugation. The enzyme released from the granules by homogenization was chromatographed on a Sephacryl S-200 column. Multiple protein peaks were recovered in the chromatogram. Assay of collagenase activity in each fraction indicated, however, that only one peak demonstrated significant collagenase activity. The enzyme activity in this peak could be markedly enhanced by incubating each fraction separately with sulfhydryl reagents. The collagenase could also be activated by a brief exposure to trypsin. However, the extent of the activation by trypsin was clearly less than that obtained with sulfhydryl reagents even though various exposure times with different trypsin concentrations were tested (Uitto, V.-J., unpublished data). Further studies demonstrated that the latter part of the chromatogram on gel filtration contained proteolytic activity against azocoll and azocasein. These fractions were also capable of activating the leukocyte collagenase which eluted earlier in the column; the characterization of this proteolytic activity is in progress. Partial purification of the leukocyte collagenase by gel filtration, therefore, allowed a separation of the enzyme from compounds which might inhibit or activate the collagenase in further experiments.

Activation of leukocyte collagenase by sulfhydryl compounds

During the purification of leukocyte collagenase a mixture of protease inhibitors, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM *p*-aminobenzamidine and 10 mM *N*-ethylmaleimide, was added to the enzyme preparation before homogenization to prevent proteolytic degradation of the enzyme. The addition of these protease inhibitors clearly increased the recovery of the enzyme activity from the leukocytes. When tested separately, it was noticed that *N*-ethylmaleimide alone gave the same enzyme recovery as the above mixture; however, addition of phenylmethylsulfonyl fluoride and *p*-aminobenzamidine resulted in the same recovery as obtained without protease inhibitors. It was further noticed that the addition of 10 mM *N*-ethylmaleimide to the supernatant of the preparation homogenized without protease inhibitors or homogenized in the presence of phenylmethylsulfonyl fluoride and *p*-aminobenzamidine markedly increased the activity of collagenase in the preparation. In subsequent studies the partially purified enzyme was incubated with varying concentrations of *N*-ethylmaleimide. The results indicated that small amounts of *N*-ethylmaleimide markedly increased the enzyme activity, and the maximal activation was achieved with about 5 mM *N*-ethylmaleimide (Fig. 1A). Another sulfhydryl reagent, *p*-aminophenylmercuric acetate, also markedly increased the collagenase activity (Fig. 1B). Under the incubation conditions used, however, *p*-aminophenylmercuric acetate maximally activated the collagenase in 0.5—1 mM concentrations. Comparison of the collagenase before and after activation revealed no significant change in the apparent molecular weight when estimated by gel filtration on Sephacryl S-200.

In further experiments, the time required for activation of the collagenase

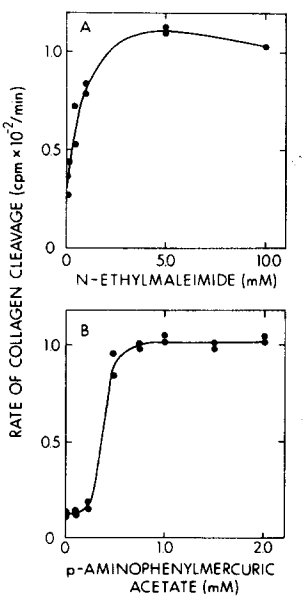

Fig. 1. Activation of leukocyte collagenase by *N*-ethylmaleimide and *p*-aminophenylmercuric acetate. Aliquots of partially purified collagenase were incubated with sulfhydryl reagents in concentrations indicated for 30 min at 25° C, and the rate of collagen cleavage was then measured.

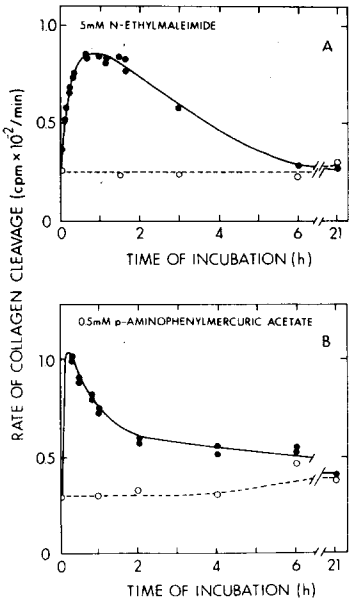

Fig. 2. Effect of the time of incubation on the activation of leukocyte collagenase by the sulfhydryl reagents. Aliquots of partially purified collagenase were incubated in the presence of 5 mM *N*-ethylmaleimide or 0.5 mM *p*-aminophenylmercuric acetate at 25°C for the time periods indicated. Parallel samples were incubated without sulfhydryl reagent. The rate of collagen cleavage was determined at the end of the incubation period. •, samples incubated in the presence of a sulfhydryl reagent; ○, control samples incubated without sulfhydryl reagents.

was studied by incubating the enzyme preparations with 5 mM *N*-ethylmaleimide or with 0.5 mM *p*-aminophenylmercuric acetate for varying time periods. The results indicated that the activation of the enzyme occurred relatively rapidly, and the maximum activation was achieved in about 30 min with *N*-ethylmaleimide, or even earlier with *p*-aminophenylmercuric acetate (Fig. 2). It was noted, however, that with increasing incubation periods the initial activation of the collagenase was lost, so that after 6 h incubation period at 25°C, in the presence of *N*-ethylmaleimide or *p*-aminophenylmercuric acetate, the enzyme activity had returned to the same levels as was observed in enzyme preparations incubated without the sulfhydryl reagents (Fig. 2). Further incubation of the enzyme with sulfhydryl reagents, even up to 21 h, did not further decrease the enzyme activity below the control level which represented about 30% of the maximal activity. Also, further addition of freshly prepared *N*-ethylmaleimide or *p*-aminophenylmercuric acetate did not affect the enzyme activity after the initial 6 or 21 h incubation.

The activation was studied in further experiments by removing the excess of the activating sulfhydryl reagent after a 30 min initial activation by dialysis against 0.2 M NaCl and 5 mM $CaCl_2$ in 0.05 M Tris-HCl (pH 7.5), at 4°C for 7 h; the control samples were dialyzed against the same buffer containing the sulfhydryl reagent. Results demonstrated that the enzyme preparation lost its initial activity at the same rate whether the dialysis buffer contained the sulfhydryl reagent or not (Table I). The enzyme activation was also studied by

TABLE I

EFFECT OF DIALYSIS ON THE ACTIVITY OF LEUKOCYTE COLLAGENASE ACTIVATED BY *N*-ETHYLMALEIMIDE (NEM) AND *p*-AMINOPHENYLMERCURIC ACETATE (*p*-APMA)

Partially purified collagenase was incubated at 25°C for 30 min with sulfhydryl reagents and then dialyzed for 7 h against 0.2 M NaCl, 5 mM $CaCl_2$, 0.05 M Tris-HCl, (pH 7.5), with or without sulfhydryl reagents. After the treatment periods, the enzyme activity was assayed. The values are expressed as radioactivity recovered in α^A and α^B peptides by SDS-polyacrylamide gel electrophoresis.

Treatment	Rate of collagen cleavage (cpm/min) × 10^{-2}
None	3.3
None	3.5
Activated with 5 mM NEM	11.5
Activated with 5 mM NEM, dialyzed vs. buffer	3.7
Activated with 5 mM NEM, dialyzed vs. NEM	4.0
Activated with 0.5 mM p-APMA	10.3
Activated with 0.5 mM p-APMA, dialyzed vs. buffer	3.8
Activated with 0.5 mM p-APMA, dialyzed vs. p-APMA	3.3

rapidly removing the *N*-ethylmaleimide or *p*-aminophenylmercuric acetate by gel filtration on Ultrogel AcA-44. The activated enzyme preparation, which chromatographed in the void volume of the gel filtration column, returned its activity to the level noted prior to activation even after the unbound sulfhydryl reagents had been removed. These results indicate, therefore, that *N*-ethylmaleimide and *p*-aminophenylmercuric acetate rapidly activate leukocyte collagenase, but the enzyme activity gradually returns to the control level.

In order to exclude the possibility that the sulfhydryl reagents might enhance the collagenase activity by reacting with the substrate, control experiments were performed. The collagen substrate was first incubated with 5 mM *N*-ethylmaleimide or 0.5 mM *p*-aminophenylmercuric acetate for 30 min, and the excess of the sulfhydryl reagent was then removed by dialysis. Subsequent incubation of the treated collagen substrate with collagenase demonstrated that the rate of collagen cleavage was no different from that observed with untreated substrate. These results suggest that the incubation with the sulfhydryl reagents does not increase the susceptibility of the substrate to collagenase cleavage.

Reversibility of the N-ethylmaleimide-induced activation of collagenase by cysteine

Previously, cysteine has been shown to inhibit leukocyte collagenase activity [2,27]. In this study, incubation of collagenase with 2 mM cysteine did not markedly activate nor inhibit the initial enzyme activity (Table II). Cysteine, however, if added to an enzyme preparation which already had been activated by *N*-ethylmaleimide, returned the activity to the control level; at least a 2-fold molar excess of cysteine over *N*-ethylmaleimide was required. This inhibition by cysteine could be further abolished by adding a 2-fold molar excess of *N*-ethylmaleimide.

TABLE II

REVERSAL OF *N*-ETHYLMALEIMIDE (NEM) INDUCED ACTIVATION OF PARTIALLY PURIFIED LEUKOCYTE COLLAGENASE BY CYSTEINE

Partially purified collagenase was incubated with *N*-ethylmaleimide at 25°C for 30 min and then further incubated for 30 min with or without cysteine in the concentrations indicated. NEM was then added for a second period of 30 min after the incubation with cysteine. After these treatments, the enzyme activity was assayed. The values are expressed as radioactivity recovered in α^A and α^B peptides by SDS-polyacrylamide gel electrophoresis.

Treatment	Rate of collagen cleavage (cpm/min) × 10^{-2}
None	0.9
None	1.1
2 mM Cysteine	0.8
2 mM Cysteine	0.9
2 mM NEM	2.4
2 mM NEM	2.8
2 mM NEM, followed by 2 mM cysteine	2.5
2 mM NEM, followed by 2 mM cysteine	2.9
2 mM NEM, followed by 5 mM cysteine	1.0
2 mM NEM, followed by 5 mM cysteine	1.4
2 mM NEM, followed by 5 mM cysteine, followed by 10 mM NEM	3.4
2 mM NEM, followed by 5 mM cysteine, followed by 10 mM NEM	3.5

Heat stability of the leukocyte collagenase

Since the activation of leukocyte collagenase by sulfhydryl reagents could involve conformational changes in the enzyme molecule, the heat stability of the enzyme activity before and after activation by *N*-ethylmaleimide was studied; all these experiments were carried out in the presence of 5 mM Ca^{2+}. The results indicated that the initial enzyme activity before any activation was relatively stable at 60°C for at least 5 min (Fig. 3). The enzyme after 1—5 min heating at 60°C could be activated by 5 mM *N*-ethylmaleimide essentially to

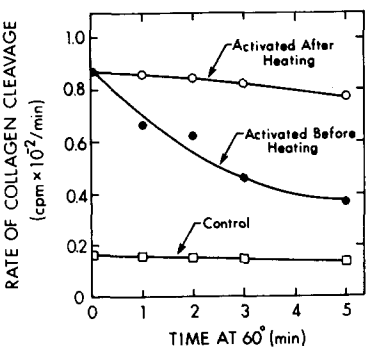

Fig. 3. Heat stability of the leukocyte collagenase before and after activation with *N*-ethylmaleimide. Aliquots of partially purified leukocyte collagenase were heated at 60° C for the time periods indicated, before or after activation with 5 mM *N*-ethylmaleimide, and the collagenase activity was then assayed. The results indicate that the control enzyme before activation (□) was relatively stable at 60° C, and it could be activated subsequent to the heating (○) to the same extent as an unheated control. In contrast, the enzyme which was activated prior to the heating by incubating the enzyme for 30 min in the presence of 5 mM *N*-ethylmaleimide at 25° C (●) lost about half of its activity during 5 min heating at 60° C.

the same extent as an unheated control. In contrast, if the enzyme was activated by incubating with 5 mM *N*-ethylmaleimide for 30 min and then subsequently incubated at 60°C, a marked decrease in the enzyme activity as a result of the 5 min heating was noted (Fig. 3). These results indicate, therefore, that the enzyme in its original form is stable at 60°C for at least 5 min and it can be fully activated by *N*-ethylmaleimide. However, once activated by a sulfhydryl reagent, the enzyme is less stable at 60°C.

Discussion

Human polymorphonuclear leukocytes contain a collagenase which plays an important role in the degradation of collagen in physiological and pathological situations [32]. The enzyme functions in the extracellular space, and therefore, the collagen degradation can be controlled either on the level of synthesis and secretion of collagenase by the cells, or the enzyme activity can be modulated extracellularly by a variety of factors. The proposed mechanisms for the extracellular control of collagenase activity include conversion of a latent proenzyme into the active enzyme by proteolytic means. or modulation of the activity of collagenase by extracellular inhibitors and activators [18—26]. Of particular interest are the observations that sulfhydryl reagents markedly stimulate collagenase activity [27—30]. In the present study, two sulfhydryl reagents, *N*-ethylmaleimide and *p*-aminophenylmercuric acetate, were shown to activate partially purified human leukocyte collagenase. The activation by the sulfhydryl reagents was a time- and dose-dependent phenomenon.

A variety of mechanisms can be proposed to explain the activation of collagenase by sulfhydryl reagents. Sulfhydryl reagents could react with the thiol groups in the active center of the enzyme, or blocking of the sulfhydryl groups in other sites of the molecule might lead to conformational changes which would render the collagenase more active. Alternatively, the sulfhydryl reagents might react with inhibitory sulfhydryl groups or inactivate inhibitors of collagenase, perhaps causing a dissociation of the enzyme-inhibitor complexes. Finally, sulfhydryl reagents might react with the substrate and render it more susceptible to collagenase digestion.

In the present study, the activation of human leukocyte collagenase by the sulfhydryl reagents was found to occur relatively rapidly but the enzyme activity gradually returned to the same level as was noted prior to activation. Also, the enzyme studied here, once activated by a sulfhydryl reagent, was less stable at 60°C than the same enzyme before activation. In previous studies, various thiol-blocking agents have been shown to increase the collagenase activity in the culture medium of a variety of rabbit tissues and cells [27—30]. The results of these studies were interpreted to suggest that the activation of the collagenase occurred as a result of dissociation of an enzyme-inhibitor complex. This conclusion was based, in part, on the observation that *p*-aminophenylmercuric acetate activated the rabbit collagenase as effectively as did enzymic treatment with trypsin. Also, the rabbit bone explants were shown to synthesize a collagenase inhibitor whose properties suggested that it was closely related to enzyme-inhibitor complexes in the culture medium.

In the present study, the activation of collagenase by the sulfhydryl reagents

was observed using partially purified leukocyte collagenase. The removal of the postgranular supernatant and chromatography of the collagenase by gel filtration allowed separation of the enzyme from possible inhibitor molecules as well as from proteolytic enzymes which activate the leukocyte collagenase. An interesting finding in this study was the reversal of the *N*-ethylmaleimide-induced collagenase activation by cysteine. Although this observation could be explained by a suggestion that cysteine provides competitive sulfhydryl groups which may displace *N*-ethylmaleimide from thiol groups of the enzyme molecule, such a possibility appears very unlikely since the covalent bond between the *N*-ethylmaleimide and the peptide-bound cysteine is remarkably stable [33]. Therefore, additional possibilities, such as the effect of cysteine on the disulfide bonds with subsequent reactivity with added *N*-ethylmaleimide, have to be investigated further. Although our results clearly indicate that sulfhydryl reagents activate human leukocyte collagenase, these observations do not disclose whether such activation involves conformational changes in the enzyme molecule or whether a dissociation of an enzyme-inhibitor complex occurs. Further physicochemical analyses are currently in progress, employing extensively purified enzyme preparations, to distinguish between these two possibilities.

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