Mechanism of Inhibition of Human Neutrophil Collagenase by Gold(I) Chrysotherapeutic Compounds

INTERACTION AT A HEAVY METAL BINDING SITE

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The mechanism of inhibition of two forms of human neutrophil collagenase (HNC) by six Au(I) compounds, some of which are used as chrysotherapeutic agents, has been investigated. The two forms of enzyme studied are active and latent HNC, the latter of which is activated by p-chloromercuribenzoate (PCMB). The effects of PCMB and Zn(II), which are normally included in the assays, on the activity of both forms of HNC and on their inhibition by these Au(I) compounds have also been studied. Zn(II) stimulates the activity of both the active and PCMB-activated latent forms of HNC up to a concentration of 50-100 μM, after which it inhibits markedly. PCMB activates latent HNC up to a concentration of 100 μM followed by inhibition at higher concentrations. Active HNC is not stimulated at PCMB concentrations below 100 μM, but is inhibited at higher concentrations. The stimulatory effects of Zn(II) and PCMB on HNC and its inhibition by PCMB are all attributable to binding at distinct sites. The inhibition of both active and PCMB-activated latent HNC by the Au(I) compounds is noncompetitive and is reversed by Zn(II). The inhibition of both forms of HNC by SKF 80544 and SKF 36914, which do not contain thiol ligands, is weak to moderate and is not influenced by the PCMB concentration. In contrast, PCMB markedly enhances the inhibition by Myocrisin, Sanocrisin, and Solganol by complexing to their thiol ligands to facilitate release of the Au(I) atom for binding to HNC. Cd(II) and Cu(II) also inhibit HNC noncompetitively, and inhibition is also reversed by Zn(II). Collectively, these data indicate that latent HNC contains a heavy metal binding site distinct from the active site at which Au(I), Cd(II), and Cu(II) bind to cause noncompetitive inhibition. Occupancy of this site by Zn(II) is characterized by retention of activity.

Comounds containing Au(I) have been used for many years to treat a variety of diseases including tuberculosis, endocarditis, syphilis, and rheumatoid arthritis (1-6). The efficacy of chrysotherapy in the treatment of rheumatoid arthritis has been confirmed in clinical trials (7-10), and Au(I) drugs continue to constitute a significant therapeutic modality. The recent development of auranofoin, an orally active Au(I) complex (11-13), has further stimulated interest in the mode of action of Au(I) drugs. While the basis for the therapeutic action of Au(I) drugs is not well understood, there is a considerable body of evidence to indicate that they, at least in part, exert their effects by altering the functions of inflammatory cells (3-5, 13-20). Of particular interest to inflammatory conditions that are associated with degradation of the extracellular matrix is the neutrophil, since it is known to contain proteinases capable of hydrolyzing collagen, elastin, and other components of connective tissue (21-25). The inhibition of neutrophil proteinases, or of the release of these proteinases from neutrophils, is a potential mechanism of action of Au(I) drugs that has been considered frequently (26-38).

The latent and active forms of human neutrophil collagenase (EC 3.4.24.7) (HNC) have recently been purified to homogeneity in our laboratory, and it was shown in an earlier report (39) that both the active and p-chloromercuribenzoate (PCMB)-activated latent forms are inhibited by six Au(I) compounds, some of which are used in chrysotherapy. It was also shown that the extent of inhibition by some of these compounds is markedly influenced by PCMB, the agent used to activate latent HNC. In view of the key role that collagenases play in collagen catabolism, the mechanism of this inhibition is of fundamental and potential clinical significance. In the present study, a series of kinetic measurements has been carried out to examine the mechanism of this inhibition in detail. In particular, the mode of inhibition of HNC by the Au(I) compounds and the effects of both PCMB and Zn(II) on the inhibition have been investigated. It is also established that inhibition of the same type is brought about by other heavy metal ions, including Cd(II), Cu(II), and Hg(II).

EXPERIMENTAL PROCEDURES

Materials—Solganol (aurothioglucose), thioglucose, Myocrisin (sodium gold thiomalate), thiomalic acid, and 1-thio-β-D-glucopyranose 2,3,4,6-tetraacetate (thioglucose tetraacetate) were purchased from Sigma; Sanocrisin (sodium gold thiosulfate) was obtained from Pfaltz and Bauer, triethylphosphine oxide from Aldrich, sodium thiosulfate, cupric sulfate, and mercuric chloride from J. T. Baker Chemical Co., and cadmium sulfate from Johnson Matthey. Auranofoin ((1-thio-β-D-glucopyranose 2,3,4,6-tetraacetato-S)(triethylphosphine)gold, SKF D-39162), chloro(triethylphosphine)gold (SKF 36914), and bis (triethylphosphine)gold (SKF 80544) were gifts of Smith, Kline and French Laboratories. Latent HNC was purified from a crude human neutrophil extract that had been treated with phenylmethanesulfonyl fluoride by sequential chromatography over reactive red-120-agarose (Sigma), an affinity matrix for HNC consisting of Pro-Leu-Gly-NH2 immobilized on CH-Sepharose 4B (40) and gelatin-Sepharose. 4B (40) and gelatin-Sepharose.

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1 The abbreviations used are: HNC, human neutrophil collagenase; GSH, reduced glutathione; BSA, bovine serum albumin; PCMB, p-chloromercuribenzoato; Tricine, N-tris(hydroxymethyl)methylglycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

K. A. Mooktiar and H. E. Van Wart, manuscript in preparation.
ASSAYS OF HNC ACTIVITY ARE Normally CARRIED OUT IN THE PRESENCE OF 50 µM ZnSO₄, SINCE THIS HAS BEEN FOUND TO STABILIZE THE ENZYME DURING PURIFICATION. IN ADDITION, 100 µM PCMB IS ROUTINELY INCLUDED IN ALL ASSAYS WITH LATENT HNC TO ENSURE THE ENZYME IS ACTIVATED BY PCMB-activated latent forms of HNC were examined earlier (42). Methods—Collagenase activity was measured by monitoring the initial rate of hydrolysis, v, of soluble 3H-acetylated rat tail tendon type I collagen (43, 44) or Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln (45). Unless stated otherwise, assays were carried out with collagen as substrate at 30 °C in 50 mM Tricine, 10 mM CaCl₂, 0.5 µM ZnSO₄, 0.2 M NaCl, pH 7.5, at a collagen concentration of 100 µg/ml. Latent HNC was activated with 0.1 mM PCMB for 30 min prior to the inhibition experiments. Samples of HNC were incubated with the Au(I) compounds in 960 µl of assay buffer for 15 min, after which the assay was started by the addition of 40 µl of collagen stock solution. The rate of hydrolysis of Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln was measured at a substrate concentration of 330 µM, as described earlier (45). Auranofin and SKF 36914 were dissolved in methanol and the inhibition studies performed so that the final methanol concentration in the assay was 2% (v/v), conditions under which HNC is over 90% active. The concentration of inhibitor that gave 50% inhibition, IC₅₀, was determined from a series of assays carried out at a fixed collagen concentration of 100 µg/ml, but variable inhibitor concentrations. The inhibition results are expressed as percentages of the activity of the control in which no inhibitor was present. For the determination of IC₅₀ values, double-reciprocal plots were carried out by measuring the initial rate of hydrolysis as a function of substrate concentration over a 10-fold range that was both above and below the Kₘ. Four plots were obtained for each inhibitor, one in the absence of the inhibitor and three in which the inhibitor concentration was at, above, and below the IC₅₀ value. Some inhibition studies were performed in alternate buffer systems, as indicated in the text.

RESULTS

In an earlier report, it was shown that the six Au(I) compounds Sanocrisin, Myocrisin, Solganol, auranofin, SKF 80544, and SKF 36914 inhibit HNC in assays carried out at a single collagen concentration (39). These Au(I) compounds can be divided into two classes. Myocrisin, Sanocrisin, and Solganol all have two thiol ligands bound to the Au(I) atom and are administered parenterally. Recently, Smith, Kline and French (SKF) laboratories have synthesized the orally active drugs SKF 39144, SKF 80544, and auranofin that have at least one triethylphosphine ligand bound to the Au(I) atom. The inhibition of two different forms of HNC by these Au(I) compounds has been investigated. The first is latent HNC, which is inactive as isolated, but which can be reversibly activated by PCMB. The second form will be referred to as active HNC, since it is already active on isolation, presumably by virtue of limited proteolysis of latent HNC that occurs during extraction from the neutrophils. Both the active and PCMB-activated latent forms of HNC were examined earlier, and it was found that the PCMB used to activate latent HNC also enhances the inhibition of active HNC by some of the Au(I) compounds. Since the inhibition of HNC by these drugs is quite potent with IC₅₀ values as low as 3.5 nM, the basis for this inhibition has been examined here in greater detail.

There are several constituents of the assay mixture required for the inhibition experiments and multiple interactions can occur between them that influence the results. In particular, assays of HNC activity are normally carried out in the presence of 50 µM ZnSO₄, since this has been found to stabilize the enzyme during purification. In addition, 100 µM PCMB is routinely included in all assays with latent HNC to ensure full and continued activation. Before examining the effects of the Au(I) compounds on HNC, the effects of Zn(II) and PCMB on the collagenase activity of both latent and active HNC were investigated. The effects of Zn(II) on the collagenase activity of both active HNC and latent HNC that had been activated with 100 µM PCMB are shown in Fig. 1. In addition, the effect of Zn(II) on the rate of hydrolysis of Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln by PCMB-activated latent HNC is shown for comparison, so that an effect of Zn(II) on collagen structure would not be mistaken for an effect on HNC. For all three reactions, activity increases with the Zn(II) concentration and reaches a maximum at a value of 50–100 µM. Further increases in the Zn(II) concentration progressively decrease activity, indicating that Zn(II) binds at two distinct sites on both forms of HNC. The similarity in the data for the active and PCMB-activated latent forms of HNC indicates that the inhibition of PCMB-activated latent HNC by Zn(II) concentrations above 100 µM is not due to displacement of the activating PCMB by Zn(II). Thus, both active and PCMB-activated latent HNC have two binding sites for Zn(II), and both are distinct from the PCMB activation site on latent HNC. Binding of Zn(II) at the tighter binding site stimulates both forms of HNC, while binding at the weaker site causes inhibition.

The dependence of the collagenase activities of both active and latent HNC on the concentration of PCMB has been investigated in the presence of 0.50 and 50 µM Zn(II). In addition, the effect of PCMB on the peptidase activity of latent HNC toward Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln in 50 µM Zn(II) has been determined. The collagenase activity of the active enzyme is independent of PCMB concentration up to a value of 0.1 mM in the presence of both concentrations of Zn(II), but the activities are uniformly higher in 50 µM Zn(II) (Fig. 2A). At concentrations above 0.1 mM, however, PCMB markedly inhibits at both Zn(II) concentrations. The collagenase activity of latent HNC is also uniformly higher in the presence of 50 µM Zn(II) at all

**Fig. 1.** Variation in the collagenase (○), peptidase (□) and peptidase (○) activities of active (○) and 0.1 µM PCMB-activated (□, ○) latent HNC as a function of Zn(II) concentration. The assays were carried out in 50 mM Tricine, 10 mM CaCl₂, 0.2 M NaCl, pH 7.5, at 30 °C at the Zn(II) concentrations indicated. The activity observed in 100 µM Zn(II) is taken as 100%.
concentrations of PCMB (Fig. 2B). At both Zn(II) concentrations, activity is stimulated as the concentrations of PCMB is increased and reaches a maximum at a value at approximately 100 μM. Above this concentration, the same marked inhibition observed for active HNC is apparent at both Zn(II) concentrations. The same behavior is observed for the peptidase activity in the presence of 50 μM Zn(II). Thus, latent HNC has two binding sites for PCMB, the tighter of which causes activation and the weaker of which causes inhibition. The latter site is also present on active HNC, while no conclusions about the former can be made from these activity data. For both latent and active HNC, the activity curves in the presence and absence of activating concentrations of Zn(II) show parallel behavior. These data strongly imply that the Zn(II) activation site is distinct from both PCMB binding sites.

With these data in mind, the effects of PCMB and Zn(II) on the inhibition of HNC by the six Au(I) compounds studied earlier were investigated. In these experiments, 0.1 mM PCMB was used to activate latent HNC, and a low (0.5 μM) concentration of Zn(II) was initially chosen for the inhibition measurements. The active or PCMB-activated latent enzymes were incubated with each Au(I) compound for 15 min at 30 °C, after which collagen was added to start the assay. No time dependence was observed for the inhibition of either form of HNC by any of these compounds. Although the assay takes 5 h, there was no evidence from the assay curves that inhibition was increasing with time. Thus, all interactions between the PCMB, Au(I) compound, enzyme, and buffer components that influenced activity were probably complete within the 15-min incubation period. The inhibition of both forms of HNC by all six Au(I) compounds is fully reversible, as demonstrated by gel filtration experiments using a G-25 column to separate the Au(I) from the protein.

Three different types of effects of PCMB on the activity versus log[I] plots have been observed which are illustrated in Fig. 3 for the inhibition of active HNC by SKF 36914, Myocrisin, and Solganol. The inhibition curves for SKF 36914 (Fig. 3A) and SKF 80544 (not shown) for active HNC are not influenced by the inclusion of either 10 or 100 μM PCMB in the assays and give PCMB-independent IC₅₀ values of 28 and 1300 μM, respectively. For both SKF 36914 and SKF 80544, the IC₅₀ values for active HNC are slightly higher than the values of 16 and 790 μM, respectively, for latent HNC obtained in the presence of 100 μM PCMB. In contrast, the inclusion of PCMB in the assay mixtures for Myocrisin and Sanocrisin facilitates inhibition markedly. In the presence of 0, 10, and 100 μM PCMB, the IC₅₀ values for the inhibition of active HNC by Myocrisin are 130, 0.40, and 0.050 μM (Fig. 3B), while those for Sanocrisin are >1,006, 0.20, and 0.063 μM, respectively. For Myocrisin and Sanocrisin, the IC₅₀ values for latent HNC measured in the presence of 100 μM PCMB are 3.5 and 11 nM, respectively. These values are both markedly lower than the values for active HNC in the presence of this same concentration of PCMB. For auranozin, no inhibition of active HNC is observed at any PCMB concentration up to the solubility limit of approximately 500 μM. The IC₅₀ value for the inhibition of latent HNC by auranozin in the presence of 100 μM PCMB, however, is 160 μM.

From these observations, it is clear that PCMB affects the inhibition of HNC by these five compounds differently. SKF 80544 and SKF 36914, which each contain a triethylphosphine ligand, inhibit active HNC with IC₅₀ values that are approximately the same in the presence and absence of PCMB. Moreover, these IC₅₀ values are almost the same as those for PCMB-activated latent HNC. This indicates that their interactions with both forms of the enzyme are direct and uninfluenced by PCMB. For these two Au(I) complexes, the inhibition is weak to moderate. On the other hand, the inhibition...
of active HNC by Myocrisin and Sanocrisin increases with increasing PCMB concentration, and potent inhibition of PCMB-activated latent HNC is observed for both in the presence of 0.1 mM PCMB. Both Myocrisin and Sanocrisin exist as thiomolate-bridged cyclic or linear polymers in solution (46–48). To account for this inhibitory behavior, the mechanism shown in Fig. 4 is proposed in which PCMB complexes with the thiol ligands to free at least one coordination site on the Au(I) for binding to HNC. In this figure, \((RSH)_2Au(I)\) represents the polymeric or oligomeric form of Myocrisin or Sanocrisin. The IC50 values for the inhibition of active HNC by Sanocrisin and Myocrisin in the presence of 0.1 mM PCMB are still higher than those for latent HNC, indicating that there are differences in the interaction of these compounds with the two forms of HNC. The failure to observe inhibition of active HNC by auranofin, which also has a thiol ligand, is probably due to the inability to measure IC50 values greater than 500 μM due to its limited solubility. Alternatively, both coordination sites of the Au(I) may be needed for binding to HNC and PCMB may not be effective at freeing the Au(I) atom from the triethylphosphine ligand on the time scale of the assays.

The effect of Solganol on the activity of HNC in the presence of variable concentrations of PCMB is shown in Fig. 3C. There is no inhibition of active HNC in the absence of PCMB, but at concentrations of 10 and 100 μM a zone of inhibition is seen at Solganol concentrations in the 10 nM to 100 μM range. At both of these PCMB concentrations, increasing the Solganol concentration initially produces inhibition, but beyond a certain Solganol concentration a second process takes place that reverses this inhibition. In view of the propensity of gold-thiolates to form polymers in solution (46–48), one possibility is that the initial loss of activity in the presence of PCMB is due to inhibition by free Au(I) as described above for Myocrisin and Sanocrisin (Fig. 3B), while the reversal of this inhibition at higher Solganol concentrations is due to a decrease in free Au(I) as Solganol polymers form. PCMB apparently facilitates the release of free Au(I) from the polymers, since increasing the PCMB concentration enhances the degree of inhibition observed at a fixed concentration of Solganol. A reexamination of the effect of Solganol on PCMB-activated latent HNC shows that a similar reversal of inhibition occurs (Fig. 3C) at higher concentrations of Solganol than used previously (39).

The possibility was considered earlier that the inhibition observed by these compounds is a result of the ligands attached to the Au(I) atom, since HNC is a metalloenzyme and many of the ligands can chelate metal ions (39). Only thio-glucose, thiomalate, and thioglucose tetraacetate inhibit appreciably at concentrations below 1 mM. Thiocissate and triethylphosphine oxide (the expected oxidation product of triethylphosphine after dissociation from Au(I)) are very poor inhibitors. Only in the case of auranofin is the IC50 value of the ligand similar to that of the Au(I) compound. Thus, at least for the other five compounds, the inhibition observed is the result of a complex between the Au(I) atom and HNC.

Another possibility that was considered was that other species could mimic the "liberating" effect of PCMB. Along these lines, Griffin and Steven (49) reported that Myocrisin inhibits trypsin only in the presence of species containing a free thiol group, such as bovine serum albumin (BSA) or N-acetyl-Cys, which were referred to as "carriers." Thus, the inhibition of active HNC by Myocrisin and Sanocrisin in the presence of BSA, Cys, and reduced glutathione (GSH) was investigated. Each of the Au(I) compounds was incubated with either 0.1 mM Cys, 0.1 mM GSH, or 0.5 mg/ml BSA in 900 μl of assay buffer for 1 h at 30 °C. Active HNC was then added and the mixture incubated for an additional 15 min before starting the assay by the addition of collagen. The results of these assays are shown in Table I and indicate that none of the potential carriers improved the inhibition by Myocrisin or Sanocrisin to any near the levels found in the presence of 0.1 mM PCMB. The largest effect was the lowering of the IC50 value of Sanocrisin by GSH from >1 mM to approximately 100 μM. Increasing the concentration of GSH to 1 mM did not increase the extent of inhibition any further. Since 100 μM PCMB lowers the IC50 value of Sanocrisin to 63 nM (39), these thiol compounds are not very effective carriers for HNC.

The inhibition of both active and PCMB-activated latent HNC by these Au(I) compounds is also influenced by the Zn(II) concentration in the assays. It was shown earlier that HNC is maximally active when 50 μM Zn(II) is included in the assay (Fig. 1). IC50 curves for the inhibition of PCMB-activated latent HNC by SKF 36914 in the presence of 0.5 and 50 μM Zn(II) are shown in Fig. 5. The IC50 value is increased 20-fold from 16 to 320 μM at the higher Zn(II) concentration, implying that Zn(II) can reverse or prevent this inhibition. To investigate this observation in greater detail, a series of assays was carried out in which PCMB-activated latent HNC was first inhibited with 75 μM SKF 36914 for 30 min, after which varying amounts of Zn(II) were added and the rate of hydrolysis of collagen measured. The percent inhibition was expressed relative to assays containing the same amount of Zn(II), but no inhibitor. Increasing the Zn(II) concentration progressively decreases the percent inhibition and, at a Zn(II) concentration of 50 μM, the inhibition is completely reversed (Fig. 6). Similar experiments were carried out for the other five Au(I) compounds, and the results showed that the inhibition by all was reversed by

![Fig. 4. Possible mechanism to explain the PCMB-assisted inhibition of HNC by Au(I) compounds with thiol ligands. The scheme shown does not imply that uncomplexed Au(I) actually exists as an intermediate, but only that its binding to HNC is facilitated by the propensity of PCMB to complex with thiol ligands.](image-url)
Collagenase Inhibition by Gold(I) Compounds

**FIG. 5.** Variation in the activity of 0.1 mM PCMB-activated latent HNC by SKF 36914 in the presence of 0.5 μM (○) and 50 μM (●) ZnSO₄. The assays were carried out in 50 mM Tricine, 10 mM CaCl₂, 0.2 mM NaCl, 0.1 mM PCMB, pH 7.5, at 30 °C at the Zn(II) concentrations indicated.

**FIG. 6.** Reversal by Zn(II) of the inhibition of 0.1 mM PCMB-activated latent HNC by 75 μM SKF 36914. The assays were carried out in 50 mM Tricine, 10 mM CaCl₂, 0.2 mM NaCl, 0.1 mM PCMB, pH 7.5, at 30 °C. The percent activity is relative to a control assay containing the same concentration of Zn(II), but no inhibitor.

**TABLE II**

Reversal by Zn(II) of the inhibition of PCMB-activated latent HNC by gold(I) compounds

<table>
<thead>
<tr>
<th>Gold(I) compound</th>
<th>Zn(II) μM</th>
<th>Activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>SKF 36914 (75 μM)</td>
<td>0.5</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>SKF 80544 (10 mM)</td>
<td>0.5</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>Auranofin (0.5 mM)</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>76</td>
</tr>
<tr>
<td>Sanocrisin (1 μM)</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>74</td>
</tr>
<tr>
<td>Myocrisin (1 μM)</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>62</td>
</tr>
</tbody>
</table>

*Expressed relative to an assay at the same Zn(II) concentration, but without inhibitor.

**FIG. 7.** Double-reciprocal plots for the hydrolysis of type I collagen by active HNC obtained in the absence (○) and presence of 0.5 (●), 1.5 (▲), and 3 mM (■) SKF 80544. The inset shows a slope replot which gives a $K_M$ value of 1.5 mM. The assays were carried out in 50 mM Tricine, 10 mM CaCl₂, 0.2 mM NaCl, 0.5 μM Zn(II), 0.1 mM PCMB, pH 7.5, at 30 °C.

Zn(II) (Table II). Much higher ratios of Zn(II) to Au(I) compound were required to reverse the inhibition by the Au(I) compounds with the lowest IC₅₀ values, consistent with a competition for binding between Zn(II) and Au(I) at a common site.

In order to determine the mode of inhibition of HNC by these Au(I) compounds and to obtain accurate values of $K_I$, we attempted to obtain double-reciprocal plots by measuring initial rates over a collagen concentration range that was varied both above (1 mg/ml) and below (0.1 mg/ml) the $K_M$ value for the reaction (0.33 mg/ml). The inhibitor concentrations were likewise varied both above and below the IC₅₀ values. These measurements proceeded smoothly for SKF 80544 and SKF 36914, and plots for the reaction of active HNC with collagen in the presence of 0.1 mM PCMB and variable concentrations of SKF 80544 are shown in Fig. 7. All of the double-reciprocal plots intersect the 1/[S] axis at a common point, indicative of noncompetitive inhibition. A
slope replot of $K_{so}/V_{max}$ versus total inhibitor concentration (50) is shown in the inset to Fig. 7 and gives a $K_l$ value of 1.5 mM. Similar data were obtained for SKF 36914, which also exhibited noncompetitive inhibition with a $K_l$ value of 48 $\mu$M (Table III).

For Sanocrisin and Myocrisin, the determination of $K_l$ values from double-reciprocal plots was complicated by interactions between these compounds and collagen. The interactions first became apparent when it was observed that the $IC_{50}$ values depended on the collagen concentration used in the assay. As the collagen concentration is increased, the $IC_{50}$ values for both compounds increased nonlinearily (Fig. 8). By comparison, the $IC_{50}$ values for SKF 36914 and SKF 80544 (not shown) were independent of collagen concentration. This dependence of $IC_{50}$ value on collagen concentration is probably due to the binding of the Au(I) atoms of Sanocrisin and Myocrisin to collagen at high collagen concentrations. Myocrisin is known to bind to and precipitate collagen from solution (51). The liberation of the Au(I) atom from its thiol ligands by PCMB probably facilitates such interactions with the collagen. As a result, the inhibition data for these Au(I) compounds at collagen concentrations greater than 100 $\mu$g/ml could not be used for the double-reciprocal plots. However, plots obtained using data in the lower substrate concentration range were satisfactory, even though the highest substrate concentration was only 0.3 $\mu$M. The plots for Sanocrisin (Fig. 9) and Myocrisin (not shown) display a clear pattern of noncompetitive inhibition. The values of $K_l$ estimated from these plots are 60 and 62 $\mu$M for Myocrisin and Sanocrisin, respectively (Table III). Since the HNC concentration employed in the assays is 0.44 nM, no significant depletion of the total inhibitor concentration by these tight binding Au(I) compounds occurs, and the use of the plot shown in Fig. 9 to estimate $K_l$ is valid. The $K_l$ values shown in Table III are in reasonably good agreement with the corresponding $IC_{50}$ values measured earlier (39), as expected for noncompetitive inhibitors.

The inhibition of HNC by the Au(I) compounds studied here and the reversal of this inhibition by Zn(II) suggest that both interact with the same metal binding site on the enzyme. Thus, the inhibition of HNC by several other metal ions, including Ag(I), Cd(II), Cu(II), and Hg(II), has been investigated. The assay buffer used was 10 mM Hepes, 10 mM Ca(NO$_3$)$_2$, 0.2 mM NaN$_3$, pH 7.5, for all ions except Hg(II). Hepes buffer was used because of its minimal tendency to chelate these metal ions (52). Nitrate salts were used in place of chloride salts to prevent precipitation of silver chloride. Above a certain critical concentration, all of these metal ions caused collagen to precipitate. The maximal concentration of metal ions that could be tested were 1, 10, and 100 $\mu$M for Cu(II), Ag(I), and Cd(II), respectively. Hg(II) caused the collagen to precipitate in the above assay buffer at micromolar concentrations. However, by using chloride as the counter ion, assays in the presence of Hg(II) could be carried out up to a concentration of 500 $\mu$M. Active HNC was inhibited in the buffers described above by Cu(II), Cd(II), and Hg(II). Double-reciprocal plots were obtained at variable concentrations of Cu(II) and Cd(II), and these results showed that inhibition was noncompetitive for both metal ions with slope replots that gave $K_l$ values of 0.26 and 1.5 $\mu$M, respectively. The $IC_{50}$ value for the inhibition by Hg(II) was 150 $\mu$M. The inhibition by all three ions was fully reversed by 50 $\mu$M Zn(II). Ag(I) did not inhibit active HNC at all at 10 $\mu$M, the maximum concentration that could be tested.

Since the publication of our earlier observations on the inhibition of HNC by Au(I) compounds, Lindy and associates (53) have reported that 1.5 mM Myocrisin activates latent HNC in 50 mM Tris-HCl, 0.2 mM NaCl, 5 mM CaCl$_2$, 1 mM glucose, pH 7.5 at 37 °C. Attempts on our part to reproduce their results have not been successful. In our hands, the addition of 1.5 mM Myocrisin to the buffer solution indicated

### Table III

<table>
<thead>
<tr>
<th>Gold(I) compound</th>
<th>$K_l$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF 36914</td>
<td>48</td>
</tr>
<tr>
<td>SKF 80544</td>
<td>1500</td>
</tr>
<tr>
<td>Myocrisin</td>
<td>0.060</td>
</tr>
<tr>
<td>Sanocrisin</td>
<td>0.062</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>0.26</td>
</tr>
<tr>
<td>Cd(II)</td>
<td>1.5</td>
</tr>
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Fig. 8. Variation in the $IC_{50}$ values for Sanocrisin (●), Myocrisin (□), and SKF 36914 (▲) measured at different collagen concentrations. The assays were carried out in 50 mM Tricine, 10 mM CaCl$_2$, 0.2 mM NaCl, 0.5 mM Zn(II), 0.1 mM PCMB, pH 7.5, at 30 °C.

Fig. 9. Double-reciprocal plots for the hydrolysis of type I collagen by active HNC obtained in the absence (○) and presence of 25 (●), 50 (▲), and 100 mM (■) Sanocrisin. The inset shows a slope replot which gives a $K_l$ value of 62 $\mu$M. The assays were carried out in 50 mM Tricine, 10 mM CaCl$_2$, 0.2 mM NaCl, 0.5 $\mu$M Zn(II), 0.1 mM PCMB, pH 7.5, at 30 °C.
results in precipitation due to complex formation between the CaCl2 and thiolate ligand of Myocrisin, and no activation of latent HNC is observed. However, incubation of latent HNC with 5 mM Myocrisin for 1 h produced an approximate 50% activation. Thus, it is possible that these Au(I) compounds can both activate and inhibit latent HNC, much the same as PCMB (Fig. 2).

Several experiments were carried out to assess the effects of the six Au(I) compounds studied here on the bacterial collagenase, \( \beta-C.\) histolyticum collagenase. The identical incubations and assays described above for HNC were performed for \( \beta-C.\) histolyticum collagenase. Only SKF 36914 was found to inhibit, giving an IC50 value of 8.2 \( \mu \)M both in the absence and presence of 100 \( \mu \)M PCMB. The IC50 value was also independent of the collagen concentration in the assay and was not reversed by Zn(II) up to a concentration of 1 mM. Thus, the pattern of the inhibition observed for \( \beta-C.\) histolyticum collagenase is markedly different from that of HNC. In contrast, preliminary experiments with human fibroblast collagenase indicate a pattern of inhibition by these six Au(I) compounds that is similar to that for HNC. Thus, the results presented here seem to be specific to human collagenases.

**DISCUSSION**

The data presented here clearly show that HNC possesses a number of different binding sites for transition metal ions and organomercurials. There are tight binding activation and weak binding inhibition sites for both PCMB and Zn(II) on latent HNC. The data presented in Figs. 1 and 2 would indicate that the two binding sites on latent HNC for PCMB and the tight binding activation site for Zn(II) are independent sites. There is also at least one binding site whose occupancy by Au(I), Cd(II), Hg(II), and Cu(II) causes inhibition, but whose occupancy by Zn(II) is characterized by retention of activity. In addition to these sites, both active and latent HNC are presumed from other studies to contain Zn(II) at the active site, a view consistent with the inhibition of the enzyme by transition metal ion chelating agents (54).

Although the precise relationship between these various sites is outside the scope of the present studies, the presence of four distinct metal binding sites on latent HNC can be inferred. The first site is at the active site where a tightly bound, intrinsic Zn(II) is thought to reside. The noncompetitive inhibition of HNC by Au(I), Cu(II), and Cd(II) indicates the existence of a second metal binding site that is distinct from the active site. The reversal by Zn(II) of the inhibition by these ions indicates that this may be the same site as the tight binding Zn(II) activation site. The inhibition of HNC by Hg(II) or high concentrations of organomercurials may also be the result of their binding to this site. This site is referred to as the extrinsic metal binding site, although it is not known whether it is empty or partially or fully occupied by a metal ion on isolation of HNC. The binding of organomercurials such as PCMB and \( p\)-aminophenylmercuric acetate to a third site produces activation of latent HNC (55). The same activation by organomercurials has been observed for human fibroblast collagenase (56). The tight binding organomercurial activation site on latent HNC is clearly a distinct site from both the active site and the extrinsic metal binding site. The inhibition by high concentrations of Zn(II) is due to binding at a fourth site and is reminiscent of other zinc metalloproteinases such as carboxypeptidase A, where the inhibition is competitive with substrate and is attributable to binding to catalytic residues at the active site (57). Three of the sites on latent HNC distinguished in the discussion above are shown schematically in Fig. 10. Since the inhibitory binding site for Zn(II) is thought to be at the active site, this site is not shown separately.

The pattern of inhibition of HNC by the Au(I) compounds studied here and the effects of PCMB on the inhibition are related to the reactivity and stability of these complexes under the inhibition conditions. Au(I) complexes are usually linear and bidentate with a preference for large, polarizable ligands (58). Ligand exchange reactions of linear Au(I) complexes with chloride and thiol ligands are labile (59, 60). Thus, the ligands present in the Au(I) complexes under study here, the ease of displacement would be expected to follow the order chloride > thiol > triethylphosphine. By this reasoning, the poor inhibition of HNC observed for SKF 80544 is attributable to its inherent unreactivity toward substitution reactions with protein ligands, none of which are preferred over triethylphosphine. SKF 36914 and auranofin would likewise not be expected to efficiently inhibit HNC if substitution of both of their ligands by protein ligands is required. Sanocrisin, Myocrisin, and Solganol are all expected to participate in ligand exchange reactions with protein ligands, but again only if the protein binding site were more thermodynamically stable.

Titration of latent HNC with Ellman's reagent indicates that it has no accessible, free thiol groups due to Cys residues. Thus, the protein ligand(s) at the Au(I) binding site on HNC is probably a nitrogen donor, such as His, that does not have a high affinity for Au(I). In this vein, the use of PCMB to complex the thiol ligands of Sanocrisin and Myocrisin has the effect of forcing their Au(I) atoms into a site on HNC that would not normally be occupied if a better donor or donor set were available.

The present study demonstrates that Au(I) compounds can be potent inhibitors of HNC under the appropriate conditions. Since cartilage is composed of type II collagen which is susceptible to attack by HNC, it is possible that inhibition of HNC, or of collagenases from other endogenous or inflammatory cells, could be an important mechanism for chrysotherapy. Given the number of factors that determines the strength of binding of Au(I) to HNC, it is extremely difficult to judge whether the inhibition of tissue collagenases by Au(I) is a reasonable possibility in vivo. Pharmacokinetic studies have shown that the Au(I) from chrysotherapeutic agents penetrates the synovial fluid where it equilibrates with serum Au(I) and can reach the concentration of 30 \( \mu \)M (5). The nature of the ligand exchange reactions that therapeutic Au(I) complexes undergo following administration is not known with certainty, but the Au(I) in serum is largely bound to albumin and globulins (5). For auranofin, in vitro experiments suggest that the thiol ligand is displaced by Cys-34 of BSA

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3 E. L. Angleton, H. Birkedal-Hansen, and H. E. Van Wart, unpublished observations.
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(61), while in vivo experiments indicate that the triethylphosphine ligand is lost as the oxide within 3 days (62). Thus, it appears that if Au(I) is to be able to bind efficiently to collagenase in vivo to prevent collagen catabolism, it would have to be mobilized from its thermodynamically stable state bound to bulk protein in serum and delivered to the restricted foci where collagenases are acting and conditions are favorable for the transfer of Au(I) to the enzyme. This study also indicates that other transition metal ions, including Cd(II), Cu(II), and Hg(II), can inhibit HNC at reasonably low concentrations, suggesting that they are also capable of mimicking the inhibition of human collagenases by Au(I).

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REFERENCES