Studies on the Active Site of Clostripain

THE SPECIFIC INACTIVATION BY THE CHLOROMETHYL KETONE DERIVED FROM α-N-TOSYL-L-LYSINE*

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SUMMARY

Clostripain is rapidly and selectively inactivated by the chloromethyl ketone derived from α-N-tosyl-L-lysine (TLCK) with an apparent second order rate constant of 8.7 × 10^-4 M^-1 sec^-1 at pH 6.5. Alkylation by TLCK occurs only with catalytically active enzyme, the rate of which is considerably retarded by the competitive inhibitors, α-N-tosyl-L-homoarginine methyl ester and benzamidine. In contrast, the chloromethyl ketone derived from α-N-tosyl-L-phenylalanine (TPCK) does not inhibit clostripain at molar concentrations 100 times greater than the effective concentration of TLCK. A very large molar excess of TPCK does inhibit the enzyme with an apparent rate constant of 40.7 M^-1 sec^-1 at pH 6.5. Other irreversible inhibitors of clostripain such as dibromoacetone and iodoacetate require molar concentrations 300 and 3000 times greater, respectively, than TLCK for equivalent inactivation. Such results are in accord with the substrate specificity of the enzyme and support an active site-directed mechanism for inhibition by TLCK. The rapidity of alkylation of the active site of clostripain by TLCK is consistent with the large kcat (643 sec^-1) observed for substrate hydrolysis (benzoylarginine ethyl ester) and the tentative identification of an essential —SH group in the catalytic mechanism. These results extend the use of TLCK in labeling the active site of a protease with high arginine specificity and offer a method for the specific irreversible inactivation of clostripain which occurs as the major proteolytic contaminant in both crude and highly purified collagenase preparations.

Clostripain (EC 3.4.4.20) is a sulfhydryl protease obtained from the culture filtrate of Clostridium histolyticum. Its substrate specificity has been demonstrated to be similar to that of trypsin in that hydrolysis is restricted to substrates containing arginine or lysine in a peptide or ester bound at the carboxyl terminus. However, specificity is more sharply defined, since hydrolysis occurs at significantly more rapid rates at arginy1 bonds, both in synthetic substrates and natural polypeptides and proteins (1). This unique specificity appears to arise from the combined effect of a higher $K_m$ and a lower $k_{cat}$ for lysyl substrates as contrasted with their arginyl homologs (2). Moreover, the preferential binding affinity for arginine is strongly supported by experiments with various alkylguanidine and alkylamine competitive inhibitors. While trypsin poorly distinguishes between the two series (3), the observed $K_i$ for the alkylguaninides (especially $n$-butylguanidine) is several orders of magnitude greater than the corresponding alkylamines (2). It is clear that the anionic binding site in clostripain thermodynamically distinguishes the delocalized positive charge of the planar guanidinium group from the static charge of the tetrahedral ammonium ion (1). A further example of the remarkable specificity of clostripain and an indication for a unique catalytic mechanism as well, is the relatively more rapid rate of hydrolysis of the trypsin-resistant arginy1proline bond than of the trypsin-sensitive lysylarginine bond in synthetic methionyllysyl bradykinin (4).

An enzymic active site can generally be considered to be composed of two functionally distinct parts, the catalytic site and the recognition or specificity site. The catalytic site comprises those groups directly involved in the bond making and breaking sequences leading to products, whereas the specificity site includes that region of the active site which is responsible for recognition and binding of the substrate. It appears that the catalytic site of clostripain may have features in common with the catalytic sites of the papain, renin, and bromelain family of sulfhydryl enzymes. On the other hand, the specificity site is more closely analogous to that of trypsin. It is, therefore, of interest to investigate the chemistry of the active site of clostripain, both with regard to detailed catalytic mechanisms and to substrate specificity. The experimental approach taken in the present investigation was that of affinity labeling. The reagent chosen was α-N-tosyl-L-lysine chloromethyl ketone, an alkyllating agent designed as an active site-directed inhibitor of trypsin, in which case it stoichiometrically reacts with a single histidine residue (5). In a similar manner, a specific alkylation of histidine in the active site of chymotrypsin occurs with N-tosyl-L-phenylalanine chloro-
methyl ketone (6). In contrast to those results, both TLCK- and TPCK alkylate the essential sulfydryl group in the active site of papain (7), ficin (8), and very likely bromelain (9). Since these sulfydryl proteases demonstrate a broader substrate specificity than either trypsin or chymotrypsin, it is of fundamental interest to compare the effects of TLCK and TPCK on the catalytic activity of a sulfydryl protease with a sharply defined substrate specificity. We wish to report the active site-directed inactivation of clostripain by TLCK and the lack of inhibition under similar conditions by TPCK.

**EXPERIMENTAL PROCEDURE**

**Materials**

TLCK, TPCK, BAE, and DTT were all products of Calbiochem. [Lys-<sup>-3</sup>H]TLCK (8.8 x 10<sup>4</sup> dpm per amole) was the kind gift of Dr. Elliott N. Shaw. Dibromochloromethane was purchased from Dajac Laboratories and used without further purification. Iodoacetic acid was obtained as the sodium salt from Sigma Chemical Company and recrystallized from 95% ethanol. Benzanilide hydrochloride was purchased from K and K Laboratories. THAME was the generous gift of Dr. Tadashi Inagami. Crude collagenase was obtained from Worthington Biochemicals.

**Methods**

**Purification and Activation of Clostripain**

Clostripain was purified from commercial crude collagenase by a modification of the procedure of Mitchell and Harrington (10). It has been necessary with recent commercial crude collagenase preparations to add an additional chromatographic step to completely remove pigment. This involves a linear gradient elution (900 ml, 0.1 M to 0.3 M sodium phosphate, pH 6.7) from a hydroxylapatite column (2.5 x 13 cm). The Sephadex G-75 enzyme fraction from the original procedure (10) is loaded onto the column in 0.05 M sodium phosphate, pH 7.8, containing 10 mM DTT at room temperature after a prior incubation (reduction) in 10 mM DTT for 5 hours at 4°. If the reduction step is omitted, the enzyme elutes in two fractions from the hydroxylapatite gradient column. In addition, recent preparations of crude collagenase have yielded enzyme with considerably lower specific activity than previously reported, unless the enzyme is activated prior to assay by incubating in 0.2 M sodium phosphate, pH 7.8, containing 10 mM DTT for 5 to 8 hours at 4°. This prolonged reduction step results in a 4-fold increase in specific activity to a maximum of 125. Clostripain solutions were stable for at least 3 days at 4° in the above reducing mixture.

**Enzyme Assay**

The standard assay employed the spectrophotometric determination of the initial rate of hydrolysis of BAE in a volume of 1 ml, containing 2.375 x 10<sup>-4</sup> M BAE, 2.5 x 10<sup>-5</sup> M DTT, and 0.1 M sodium phosphate buffer, pH 7.8. Initial rates were determined at 35° with a Gilford model 2000 recording spectrophotometer with specific activity expressed as micromoles of BAE hydrolyzed min<sup>-1</sup> mg<sup>-1</sup> of protein (11). Protein concentration was determined by dry weight or by a modified microbiuret reagent (12) using bovine serum albumin as standard. All biuret values were corrected, since this determination gave results 1.6 times higher for clostripain concentration than determination by dry weight. Molar concentrations of clostripain were calculated using a molecular weight of 30,000 (10).

**TLCK Inhibition Studies**

**Kinetic Methods**—Reactions were carried out at room temperature after activating clostripain at a concentration of 2.8 x 10<sup>-6</sup> M in 0.2 M sodium phosphate buffer, pH 7.8, containing 10 mM DTT overnight at 4°. Aliquots of 10 μl were removed and added to 0.49 ml of 0.05 M sodium phosphate, pH 6.5. Immediately after mixing, 0.25 ml of an appropriate concentration of TLCK in H<sub>2</sub>O was added. Aliquots of 50 to 100 μl were removed for assay with BAE.

Although clostripain at high concentrations (1 mg per ml) under reducing conditions is relatively stable, the conditions employed in these studies (high dilutions of enzyme and reducing agent to approximate enzyme kinetics and minimize reducing agent alkylation, respectively) resulted in activity loss in the control samples varying from 0 to 30% (usually about 20%), necessitating the computation of esterase activity loss as percentage of control activity under identical conditions.

*<sup>[3H]</sup>TLCK Incorporation* —A typical reaction was performed by activating the enzyme (0.067 pmoles) in 10 ml of 0.2 M sodium phosphate, pH 7.8, containing 10 mM DTT overnight at 4° (stable at least for 3 days under these conditions). After thermal equilibration at room temperature, the pH of the solution was lowered to pH 6.5 by the addition of approximately 0.8 ml of 1 N HCl. Then 0.25 ml of a solution of *<sup>[3H]</sup>TLCK in H<sub>2</sub>O (0.281 μcules) was added to the stirred enzyme solution. After about 5 min, 10-μl aliquots of the reaction mixture were assayed using the standard BAE assay. In all cases, inhibition was 94% or greater.

The reaction mixtures were dialyzed exhaustively against distilled H<sub>2</sub>O or 0.05 M sodium phosphate, pH 6.7. Scintillation counting was performed in Aquasol (New England Nuclear) using a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency of each sample was determined by the use of *<sup>[3H]</sup>toluene (New England Nuclear) as an internal standard.

**Titration of Enzymatic Activity with TLCK**—Clostripain was activated at a concentration of 0.25 mg per ml in 0.2 M sodium phosphate, pH 6.5, containing 10 mM DTT overnight at 4°. The specific activity of the enzyme activated in this manner was determined in the standard assay at pH 7.8. In addition, an aliquot of the pH 6.5 activated enzyme was diluted 1:100 into 0.2 M sodium phosphate, pH 7.8, 10 mM DTT buffer, and after 5 hours was assayed in the standard assay to determine the difference in maximal specific activity of the enzyme activated at pH 6.5 versus pH 7.8.

For the titration of enzymatic activity, 10-μl aliquots of TLCK solutions in H<sub>2</sub>O were added to 0.3 ml of the pH 6.5 activated enzyme solution. Aliquots of 10 μl were removed at 5, 15, and 30-min intervals to insure completeness of reaction, and added to 0.29 ml of 0.2 M sodium phosphate, pH 7.8, containing 10 mM DTT. Assays were performed in duplicate after overnight incubation at 4°. An appropriate control sample was included. Final concentrations in the reaction mixtures were 5.4 x 10<sup>-4</sup> M enzyme and 1 x 10<sup>-4</sup> M to 1.2 x 10<sup>-4</sup> M TLCK.

Titration curves were also carried out at pH 7.8 in a similar manner to those described for the pH 6.5 titrations except that the enzyme was activated, and reactions performed, at pH 7.8.

**Reaction of Crude Collagenase with TLCK**—Titration of
clostripain in crude collagenase was performed at pH 7.8 as described above after activation of the sample (2.0 mg per ml by weight) in 10 mM DTT for 5 hours at 4°C. In this case, however, the molar concentration of active clostripain was calculated from the total units of activity and an assumed maximal specific activity of 350 for fully active clostripain. The basis for this calculation will be considered in the text.

Reaction of crude collagenase with [3H]TLCK was carried out at pH 6.7 and 7.8, each giving similar results. In both cases, the crude enzyme was activated in 0.1 M sodium phosphate, pH 7.8, containing 10 mM DTT for 11 hours at room temperature. The molar concentration of clostripain in each case was calculated on the basis of a specific activity of 350 for fully active clostripain.

Inactivation with iodoacetate was carried out in an identical manner to that described for TLCK except acetone or methanol. Inactivation with iodoacetate was carried out at pH 6.50. Respective control samples received 10 μl of either 0.1 M NaH2PO4. Then 1 ml of a 1.14 mg per ml solution of [3H]TLCK in H2O was added with stirring. Aliquots were removed before and 15 min after addition of [3H]TLCK for assay with BAE. Final reaction concentrations were 7.44 × 10⁻⁶ M clostripain, 3.25 × 10⁻⁵ M [3H]TLCK, and 5 × 10⁻⁶ M DTT. Reaction at pH 7.8 was carried out by addition of [3H]TLCK directly to an activated solution of crude collagenase (0.5 g in 25 ml) without pH adjustment. Four successive 0.25-ml additions of 0.07 mg per ml of [3H]TLCK in H2O were made at about 5-min intervals giving final concentrations of 1.53 × 10⁻⁶ M clostripain, 4.9 × 10⁻⁵ M [3H]TLCK, and 1 × 10⁻⁵ M DTT. In both cases the entire reaction mixtures were directly applied to columns for purification of [3H]TLCK-clostripain as described for the purification of active enzyme. Radioactivity was eluted in the characteristic positions of nonalkylated clostripain on both Sephadex G-75 and hydroxylapatite as single, symmetric peaks.

Inhibition of Clostripain by TPCK, Dibromoacetone, and Iodoacetate—Reactions were carried out in a similar manner as those described for TLCK under "Kinetic Methods" except that 10 μl of an appropriate concentration of TPCK in methanol or dibromoacetone in acetone was added after dilution of the activated enzyme with 0.73 ml of 0.05 M sodium phosphate buffer, pH 6.50. Respective control samples received 10 μl of either acetone or methanol. Inactivation with iodoacetate was carried out in an identical manner to that described for TLCK except that all reactions were performed in the dark in an argon atmosphere.

Collagenase Assay—Collagenase activity was assayed (13) against gelatinol at 20°C in 0.1 M sodium citrate phosphate buffer, pH 6.3, in a low shear Ostwald viscometer with a water outflow time of 36 sec.

RESULTS

Inhibition of Clostripain by Alkylating Reagents—At an approximately 2-fold molar excess of TLCK to enzyme protein concentration, TLCK rapidly and irreversibly inhibits the enzyme esterase activity while a 100-fold molar excess of TPCK fails to inhibit the enzyme over an extended period of time (Fig. 1). This specificity is comparable to that found with trypsin, which was not inhibited by TPCK under conditions which completely inhibited chymotrypsin in 2.5 hours (6). Conversely, chymotrypsin was unaffected by a 58-fold molar excess of TLCK (5). However, at an extremely large molar excess, TPCK does inactivate clostripain. This is shown in Table I, which compares the inhibition of clostripain by various alkylating reagents on the basis of the molar concentration of inhibitor required to produce

![Figure 1](http://www.jbc.org)

**FIG. 1.** Effect of TLCK and TPCK on the enzymatic activity of clostripain. Clostripain is at a concentration of 3.75 × 10⁻⁴ M. Reactions were carried out in 0.05 M sodium phosphate, pH 6.5, in the presence of 1.1 × 10⁻⁴ M DTT. Curve 1, 6.0 × 10⁻⁵ M TLCK; Curve 2, 1.5 × 10⁻⁵ M TLCK; Curve 3, 1.5 × 10⁻⁴ M TPCK.

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative %I0%</th>
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<tr>
<td>TLCK</td>
<td>1.0</td>
</tr>
<tr>
<td>TPCK</td>
<td>2000</td>
</tr>
<tr>
<td>Dibromoacetone</td>
<td>300</td>
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<tr>
<td>Iodoacetic acid</td>
<td>3000</td>
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* Relative %I0% is defined as the molar concentration of inhibitor required to produce 90% inhibition in a period of 35 min, relative to TLCK.

**TABLE II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Inhibition</th>
<th>Ratio of TLCK to TPCK</th>
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<tr>
<td></td>
<td>TLCK</td>
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</tr>
<tr>
<td>Bromelain*</td>
<td>14.5</td>
<td>66.6</td>
</tr>
<tr>
<td>Papain*</td>
<td>2.3</td>
<td>37.4</td>
</tr>
<tr>
<td>Ficin*</td>
<td>32.9</td>
<td>61.2</td>
</tr>
<tr>
<td>Clostripain*</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* Data from Murachi and Kato (9). These enzymes were incubated with a 10-fold molar excess of TPCK or TLCK at pH 7.0 for 30 min at 30°C. Enzyme concentrations were: bromelain, 6.5 × 10⁻⁵ M; papain, 3.08 × 10⁻⁴ M; ficin, 1.33 × 10⁻⁵ M. Inhibition of clostripain (3.75 × 10⁻⁵ M) by 0.0 × 10⁻⁴ M TLCK and 1.5 × 10⁻⁴ M TPCK in 30 min at pH 6.5 and 25°C.

90% inhibition in 35 min, relative to TLCK. These results indicate a markedly restricted specificity with TLCK, even when compared with the chemically more reactive dibromoacetone or iodoacetate.

Table II compares the extent of inhibition of sulfhydryl proteases by TLCK and TPCK during a 30-min period, indicating a
greater reactivity of TLCK with all of these enzymes. This enhanced reactivity of TLCK is not the result of any inherent chemical differences in reactivity of the two α-haloketones, since under identical conditions, the rates of reaction of TLCK and TPCK with cysteine are the same (9). Rather these differences can be attributed to the substrate specificities of these enzymes. Bromelain (14) as well as papain and ficin (15, 16) catalyze the hydrolysis of peptide-bound basic amino acids more rapidly than phenylalanine bonds, although the specificity is not nearly as restricted as is the case for clostripain. Thus, the restricted substrate specificity of clostripain as contrasted to the other sulfhydryl proteases is reflected in its inhibitor specificity (Table II).

Effect of Competitive Inhibitors of Inactivation of Clostripain by TLCK—Both THAME and benzamidine are effective competitive inhibitors of clostripain. In the absence of inhibitors Lineweaver-Burk plots (Fig. 2) demonstrate typical kinetics without evidence of substrate activation or inhibition phenomena yielding a $K_{m(app)}$ of $2.35 \times 10^{-4} \text{ M}$ and a $k_{cat} = 643 \text{ sec}^{-1}$ based on catalytically active enzyme (determined by TLCK titration). Fig. 2A illustrates the Lineweaver-Burk plot for BAE hydrolysis in the presence of THAME, from which an average $K_i$ for THAME of $2.05 \times 10^{-4} \text{ M}$ was obtained. It is interesting that THAME is a substrate for trypsin with a $K_{m(app)} = 3.3 \times 10^{-4} \text{ M}$ and $k_{cat} = 4.0 \text{ sec}^{-1}$ (17). Thus, it appears that the active site of clostripain is more rigid than is that of trypsin, the extra methylene unit not accommodated in a manner acceptable for hydrolysis, although binding is still quite good. Benzamidine is an efficient competitive inhibitor of trypsin with a $K_i$ of $1.84 \times 10^{-4} \text{ M}$ (18). In view of the similarity in substrate specificity of clostripain and trypsin, it is not surprising that benzamidine is also a competitive inhibitor ($K_i = 4.29 \times 10^{-4} \text{ M}$) of clostripain as shown in Fig. 2B.

Both THAME and benzamidine, at concentrations equal to or greater than their respective $K_i$ values, are very effective in protecting the active site of clostripain from reaction with TLCK as shown in Fig. 3. This provides strong evidence that TLCK reacts specifically at the active site of clostripain to bring about inhibition.

If TLCK reaction occurs with formation of an inhibitor-enzyme complex according to the following equation

$$E + I \xrightarrow{K} EI \xrightarrow{k_2} P$$

the apparent second order rate constant for reaction under conditions where $I \ll K$ is given (19)

$$k_{obs} = \frac{k_2}{K}$$

In the presence of a competitive inhibitor ($I$) with a binding constant $K_i$
Thus, from the ratio of the observed second order rate constants (see "Second Order Rate Constants of Clostripain Inactivation") for reaction of clostripain with TLCK in the presence and absence of a competitive inhibitor, the $K_i$ of the competitive inhibitor can be calculated. In the presence of $1.33 \times 10^{-4}$ M THAME the ratio of the observed rate constant was determined to be 7.18, from which $K_i$ of $2.15 \times 10^{-4}$ M can be calculated. This inhibitor constant is in excellent agreement with the value of $2.05 \times 10^{-4}$ M benzamidine was 1.98, from which a $K_i$ of $4.08 \times 10^{-4}$ M was calculated. Again this is in good agreement with the value of $4.28 \times 10^{-4}$ M, determined by competition with BAE hydrolysis. These results provide quantitative evidence that reaction of TLCK with clostripain requires an unoccupied active site.

**Stoichiometry of TLCK Inhibition of Clostripain**—As illustrated in Curve 2 of Fig. 1, when TLCK is present in less than apparent molar equivalence to enzyme protein, inhibition is still nearly complete in 44 min. This inhibition of clostripain by less than an equimolar quantity of TLCK is apparently the result of the presence of catalytically inactive clostripain. The stoichiometry of purified clostripain inactivation by TLCK was determined by titration with unlabeled TLCK as well as by incorporation of tritiated TLCK of known specific activity. Both methods yielded identical results, as documented in Table III. Based on the specific activity at the time of TLCK inactivation, preparations of maximum specific activity incorporate approximately 0.4 moles of TLCK per 50,000 g of protein. The failure to achieve a 1:1 molar stoichiometry does not appear to be the result of incomplete reduction of potentially active enzyme by DTT at pH 7.8, since the more rigorous conditions of NaBH₄ (0.17 M) reduction at 0° produces a more rapid rate of activation (complete in 20 min) but to a level of activity identical with that resulting from YlTT activation even after 60 min. Other possible explanations would include impure clostripain preparations, an erroneous molecular weight constant, or the presence of catalytically inactive enzyme not amenable to stringent reduction procedures (NaBH₄). The original procedure for the purification of clostripain from commercial sources (10) yielded an enzyme of approximately 50,000 daltons with apparent homogeneity as assayed by sedimentation velocity and equilibrium ultracentrifugation, immunodiffusion and immunoelectrophoresis, and disc electrophoresis at both an acidic and alkaline pH. A second hydroxylapatite column was employed in the present purification as an added step to remove small amounts of contaminating pigment present in the current crude commercial prep which co-elute with clostripain. This minor additional step has resulted in preparations of equal or slightly higher specific activity after sulfhydryl reduction. However, the extreme lability of clostripain to oxidative inactivation allows this criticism only qualitative significance. Thus, as a further indication of homogeneity, sodium dodecyl sulfate gel electrophoresis (20, 21) yielded a single electrophoretic band consistent with a molecular weight of approximately 45,000 daltons. Since the clostripain preparations used in these experiments appear to be homogeneous with regard to molecular species and to possess the reported molecular weight, the only alternative appears to be a substantial quantity of catalytically inactive enzyme in our best purified preparations.

**Stoichiometry of Inhibition of Clostripain in Crude Collagenase with TLCK**—The molar stoichiometry for "maximally" active clostripain presented in Table III indicates that enzyme with the highest specific activity as yet attainable is only about 40% active. From this value and the direct relationships between catalytic activity and molar stoichiometry for inhibition by TLCK (Table III), the specific activity of fully active clostripain could be reasonably assumed to be about 2.5-fold the values found. Using this assumed specific activity, the molar concentration of clostripain in an activated sample of crude collagenase could be determined. Fig. 4 shows the results of such a titration of active clostripain with TLCK at pH 7.8. The finding of a stoichiometry of 1.1 moles of TLCK per mole of active enzyme, calculated in this way, is good evidence that no other major TLCK reactive species exist in crude collagenase. Since this titration is only a

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**Table III**

<table>
<thead>
<tr>
<th>Method</th>
<th>% Original specific activity</th>
<th>TLCK inhibition</th>
<th>Corrected for specific activity</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>moles TLCK/10,000</td>
<td>pH</td>
</tr>
<tr>
<td>PH/TLCK incorporation</td>
<td>100</td>
<td>0.38</td>
<td>0.38</td>
</tr>
<tr>
<td>PH/TLCK incorporation</td>
<td>61</td>
<td>0.23</td>
<td>0.38</td>
</tr>
<tr>
<td>TLCK titration</td>
<td>67</td>
<td>0.25</td>
<td>0.37</td>
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<tr>
<td>TLCK titration</td>
<td>67</td>
<td>0.25</td>
<td>0.37</td>
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**Fig. 4.** Titration of BAE activity in crude collagenase with TLCK. Reactions were carried out in 0.1 M sodium phosphate, pH 7.8, and 10 mM DTT. Clostripain concentration was $2.0 \times 10^{-4}$ M based on a specific activity of 350 (see text).
Stoichiometry of TLCK inhibition of clostripain in crude collagenase

<table>
<thead>
<tr>
<th>Method</th>
<th>% Inhibition in crude collagenase</th>
<th>Mols TLCK/mole purified inactivated clostripain</th>
<th>pH</th>
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<tr>
<td>[3H]TLCK incorporation</td>
<td>94</td>
<td>0.63 molar/mole</td>
<td>6.7</td>
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<tr>
<td>[3H]TLCK incorporation</td>
<td>98</td>
<td>0.60 molar/mole</td>
<td>7.8</td>
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<tr>
<td>[3H]TLCK incorporation and</td>
<td>98</td>
<td>0.70 molar/mole</td>
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<td>isoelectric focusing</td>
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Fig. 5. Second order rate plot for reaction of TLCK with clostripain. Clostripain (a), 9.0 X 10^{-7} M as determined by TLCK titration; TLCK (b), 1.5 X 10^{-8} M; DTT, 1.1 X 10^{-4} M; 0.06 M sodium phosphate, pH 6.5.

The foregoing observations support the concept of an active site directed mechanism for inhibition of clostripain by TLCK. Thus, inhibition by TLCK occurs at much lower concentrations than the chemically more reactive dibromoacetone or iodoacetate. Moreover, the ability of the competitive inhibitors, benzamidine and TPCK, with inhibition constants for inhibition of clostripain by TLCK (k(b) = 8.65 X 10^{-4} M^{-1} sec^{-1}) and TPCK (k(b) = 4.67 M^{-1} sec^{-1}) clearly reflect the remarkable specificity of the enzyme and the reactivity of the active site nucleophile of clostripain.

Collagenase Activity following TLCK—A 2 molar excess of TLCK results in a rapid inactivation of clostripain in purified preparations (Fig. 1) and in the crude starting material. Viscometric collagenase assays in the latter revealed no diminution of collagenolytic activity. Moreover, no [3H]TLCK is incorporated in the collagenase moiety.

Table IV

Second Order Rate Constants of Clostripain Inactivation—Rate data for TLCK inhibition of clostripain were computed and plotted as a second order reaction as shown in Fig. 5. This plot is for reaction at an initial TLCK concentration of 1.5 X 10^{-4} M and an actual “active” enzyme concentration of 9.0 X 10^{-4} M, as determined by titration with TLCK using a 1:1 molar stoichiometry (i.e. catalytically active enzyme). Reaction of TPCK at 3.0 X 10^{-4} M with clostripain at 9.0 X 10^{-4} M (as determined by TLCK titration) followed good pseudo-first order kinetics through 75% inhibition but then deviated from linearity. The pseudo-first order rate constant was calculated from the linear portion of the plot and converted to a second order rate constant by dividing by the TPCK molar concentration. The second order rate constants determined for reaction of clostripain with TLCK (k(b) = 8.65 X 10^{-4} M^{-1} sec^{-1}) and TPCK (k(b) = 4.67 M^{-1} sec^{-1}) clearly reflect the remarkable specificity of the enzyme and the reactivity of the active site nucleophile of clostripain.
specific activity of fully active clostripain and therefore of the molar concentration of active enzyme in crude collagenase. This calculated molar concentration of clostripain in crude enzyme when titrated with TLCK yields a 1:1 stoichiometry indicating that clostripain is the only reactive species. When TLCK titration is carried out at pH 6.5, the residual clostripain activity remaining after reactivation at pH 7.5 is identical with the specific activity differential between enzyme activated at pH 6.5 versus pH 7.8. These direct relationships between enzymatic activity and incorporation of, or titration by, TLCK demonstrate that reaction occurs only with an enzymatically active functional group which is essential to the catalytic mechanism of the enzyme. Therefore, the possibility that TLCK reacts with a group near the active site with loss of enzymatic activity resulting from conformational perturbations or steric interference with catalysis seems to be remote.

An important corollary of the work on labeling the active site of clostripain with TLCK has been the use of TLCK titration to determine the normality of active enzyme. With the knowledge of the actual molar concentration of active enzyme it has been possible to determine $k_{cat}$ for clostripain-catalyzed hydrolysis of BAE and to directly compare the equivalent kinetic constants of mum between pH 5.5 and 8, with a negative inflection at about 8.7. Therefore, the unionized form of this group, probably a cysteine as the site of alkylation in clostripain, unusual in its capacity to differentiate the guanidinum group from the ammonium group, but the catalytic site has unusual features which render it into a more highly active state than the catalytic sites of either trypsin or papain.

A further indication of an unusually reactive active site configuration is provided by the large second order rate constant of $8.7 \times 10^4$ M$^{-1}$ sec$^{-1}$ for alkylation by TLCK at pH 6.5. Considering the marked preference by clostripain for arginyl over lysyl residues in susceptible substrates and inhibitors (1), the large second order rate constant found for the TLCK reaction with clostripain may prove to be relatively small as compared with arginyl alkylating agents when these become available. Table VI compares rate constants for similar reactions of TLCK and TPCK with other enzymes and with cysteine. The larger apparent second order rate constants for papain and clostripain compared with those of trypsin and chymotrypsin are consistent with the greater nucleophilicity of a thiol over the imidazole group, suggesting cysteine as the site of alkylation in clostripain as in the other sulfhydryl proteases. Although at a fixed concentration of substrate, the pH-initial rate profile for clostripain BAE hydrolysis in bell-shaped with a maximum at pH 7.4 (1), Cole and Inagami (2) have found that the log $V_{max}$ profile for clostripain-catalyzed hydrolysis of BAE exhibits a broad maximum between pH 5.5 and 8, with a negative inflection at about pH 8 corresponding to the ionization of a single group with $pK_a$ of 8.2. Therefore, the unionized form of this group, probably a sulfhydryl, appears to be responsible for catalysis of BAE hydrolysis. Alkylation of chymotrypsin by TPCK (19) and of trypsin by TLCK (5) both demonstrate bell-shaped pH-rate constant profiles with maxima corresponding to those for hydrolysis of their respective substrates. On the other hand, a simple S-shaped pH-rate constant profile with $pK_a$ of 8.28 was observed for alkylation of papain by TPCK (24), indicating in this case that reaction occurs only with a thiolate anion, in contrast to acylation of the unionized form of this group by substrates.

The site of alkylation by TLCK has tentatively been identified as the thiol group of a cysteine residue. This is based on the requirement for reduced enzyme both for hydrolysis of BAE as well as alkylation by TLCK, by the remarkably rapid rate of in-

### Table V

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{m(app)}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_{m(app)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.0043</td>
<td>14.6</td>
<td>3205.0</td>
</tr>
<tr>
<td>Papain</td>
<td>12.2</td>
<td>15.3</td>
<td>1.25</td>
</tr>
<tr>
<td>Clostripain</td>
<td>0.235</td>
<td>643.3d</td>
<td>2738.0</td>
</tr>
</tbody>
</table>

- *Data from Inagami and Sturtevant (22); 0.025 M CaCl$_2$, pH 8.0.
- † Data from Whitaker and Bender (23); 0.2 M sodium acetate, pH 5.79.
- ‡ 0.1 M sodium phosphate, pH 7.8, 5 mM DT.
- § Based on determination of enzyme normality by TLCK titration (see text).

### Table VI

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>$k_2$</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>TLCK</td>
<td>5.6</td>
<td>6.0</td>
<td>(5)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>TPCK</td>
<td>0.79</td>
<td>6.0</td>
<td>(6)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>TPCK</td>
<td>11.2</td>
<td>7.0</td>
<td>(19)</td>
</tr>
<tr>
<td>Papain</td>
<td>TPCK</td>
<td>67.9</td>
<td>6.0</td>
<td>(24)</td>
</tr>
<tr>
<td>Papain</td>
<td>TPCK</td>
<td>1110.0</td>
<td>6.5</td>
<td>(24)</td>
</tr>
<tr>
<td>Clostripain</td>
<td>TPCK</td>
<td>8.65</td>
<td>6.0</td>
<td>(7)</td>
</tr>
<tr>
<td>Clostripain</td>
<td>TPCK</td>
<td>46.7</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>TLCK</td>
<td>6.75</td>
<td>6.57</td>
<td></td>
</tr>
</tbody>
</table>

* $K_{(1m)}$ is the maximum $k_2$ obtainable.
activation with TLCK, by kinetic arguments originating in an observed high $k_{cat}$ and $V_{max}$-pH profile (2), and by preliminary structural evidence identifying cysteine as the alkylated residue (25). Moreover, it would appear that the configuration of the active site of clostripain is such as to render the unionized form of the sulfhydryl group present into a highly active state, very susceptible to alkylation by TLCK and highly effective in the catalysis of BAE hydrolysis. Although this perhaps can be attributed in part to hydrogen bonding with an imidazole group as in trypsin, chymotrypsin (15, 26), and possibly papain (27, 28), there must be special features of the active site not present in papain which impart a high degree of nucleophilicity to the thiol group. Perhaps the extreme ease of air oxidation of the enzyme may be a direct consequence of this special configuration.

Collagenase preparations of various states of purity are utilized extensively in widely varied areas of biochemical research. For example, crude collagenase is often used to remove cells from surfaces in tissue culture while highly purified collagenase is often employed as a specific molecular probe of either collagen structure or biosynthesis, or both. Clostripain is a frequent contaminant of highly purified collagenase preparations (29), and when preserved in collagenase preparations being used in molecular probe investigations involving limit incubation conditions, its use can lead to erroneous conclusions. Thus, the stoichiometric inhibition of clostripain in crude collagenase by TLCK as well as the complete absence of radioactivity associated with the collagenase fraction after inhibition of crude collagenase with [3H]TLCK offer a highly specific and irreversible method for the elimination of clostripain activity.

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