Evidence for an active-center cysteine in the SH-proteinase α-clostripain through use of *N*-tosyl-L-lysine chloromethyl ketone

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The rapid reaction of α-clostripain with tosyl-L-lysine chloromethyl ketone results in a complete loss of activity and in the disappearance of one titratable SH group whereas the number of histidine residues is not affected. Tosyl-L-phenylalanine chloromethyl ketone and phenylmethylsulfonyl fluoride have no effect on the catalytic activity. From the molar ratio and under the assumption of 1:1 molar interaction, the fully active enzyme has a specific activity of 650–700 units/mg [twice the value proposed by Porter et al. (J. Biol. Chem. 246 (1971) 7675–7682)]. Partial oxidation makes it experimentally impossible to attain this maximal value.

α-Clostripain *Cysteine proteinase* *Active site*

1. INTRODUCTION

Clostripain (EC 3.4.4.20) is a sulfhydryl proteinase isolated from the culture filtrate of *Clostridium histolyticum* with a highly limited specificity directed at the carboxyl bond of arginyl residues in proteins and in synthetic substrates [1–4]. According to the specificity, clostripain is close to trypsin-like enzymes; on the other hand, its catalytic site is that of a SH-proteinase.

In [4], we isolated a highly active form of clostripain: α-clostripain of spec. act. 550–600 units/mg. Our preliminary study on the reaction of this proteinase with TLCK supported the hypothesis in [5] suggesting that the inhibition of activity by TLCK was due to the modification of a thiol group in an analogous way with other cysteine proteinases such as papain [6] and ficin [7]. Recently [8], we elucidated the amino acid sequence around this accessible thiol group after labelling with radioactive iodoacetic acid.

We investigate here the chemistry of the active site of α-clostripain by a detailed study of the reaction of α-clostripain with TLCK. It is shown that TLCK reacts with the sulfhydryl group of α-clostripain and that there is no reaction with histidine residues.

Abbreviations: TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; BAEE, α-*N*-benzyl-L-arginine ethyl ester; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); p-NO_2-ZACK, *N*-α-*p*-nitrobenzyloxycarbonyl-L-arginine chloromethyl ketone

2. MATERIALS AND METHODS

Culture filtrates of *C.histolyticum* were obtained from Institute Pasteur Production. BAEE was obtained from Fluka. DTT (Cleland's reagent) and PMSF were from Sigma and TPCK from Merck. DTNB was purchased from Pierce. Ultrogel AcA 202 was obtained from IBF. All other chemicals used were of analytical grade of the highest quality available.

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2.1. Enzyme purification

α-Clostripain was isolated from the culture filtrate of *C.histolyticum* as described in [4]. Instead of lyophilisation, the final solution of the pure enzyme was distributed into tubes, flushed with nitrogen, then frozen.

2.2. TLCK inhibition studies

Reactions were carried out at room temperature or 4°C. In a typical experiment, α-clostripain was activated in 50 mM Tris–HCl buffer containing 10 mM DTT for 1 h at 4°C. Exogenous reducing agent was then eliminated by passage over an AcA 202 (0.9 × 20 cm) column equilibrated and eluted with 0.1 M Tris–HCl buffer (pH 7.0). Immediately after the removal of DTT, an appropriate concentration of TLCK in H_2O was added to the aliquots of this activated enzyme solution. At various times during the course of the reaction, aliquots were assayed for residual activity with BAEE and sulfhydryl content by spectrophotometric titration.

For the study of the effect of pH on inhibition by TLCK, the buffers used were acetate (pH 3.5–5.8), phosphate (pH 5.8–7.8) and borate (7.5–9.6). The total ionic strength was maintained at 0.3 by addition of KCl.

2.3. Enzyme assay

The standard assay of α-clostripain was based on the spectrophotometric determination of the initial rate of hydrolysis of BAEE using 2-ml aliquots of 50 mM Tris–HCl, 50 mM $CaCl_2$ buffer (pH 7.4) containing 0.75 mM BAEE without DTT. Initial rates were determined with a Zeiss PMQ-11 recording spectrophotometer at 25°C. A molar absorption difference of 1150 $M^{-1} \cdot cm^{-1}$ was used in all calculations.

2.4. Tritration of the thiol groups in the enzyme with DTNB

Thiol estimations were calculated on the basis of a molar absorbance of 13 600 for 5-carboxy-4-nitrothiophenol at 412 nm [9]. The A_{412} was read against the blank treated in parallel but with no protein. The production of 5-carboxy-4-nitrothiophenol was measured and recorded with a Perkin-Elmer model 550 S recording spectrophotometer (sensitivity of the recorder permitted us to record an absorbance of 0.050 or 0.100 as full scale).

Titrations of free thiol groups in the native or modified enzyme were carried out at pH 7.0 as follows: to one cuvette, 0.2 ml enzyme solution and 0.8 ml of 0.1 M buffer (pH 7.0) (Tris/HCl or phosphate) were added. The control cuvette contained 1 ml of the same buffer. 0.020 ml of 10 mM DTNB was added to both cuvettes and the increase in A_{412} was recorded.

2.5. Amino acid analyses

Amino acid analyses were performed on a Beckman Multichrom B apparatus equipped with an Icap 10 Integrator using a single column procedure [10]. Samples were hydrolyzed in 6 N HCl for 20 h at 110°C under vacuum. Half-cystine was estimated as cysteic acid after performic acid oxidation [11].

3. RESULTS

3.1. Inhibition of α-clostripain by alkylating reagents

At 4°C, pH 7.0 and a TLCK:enzyme molar ratio of 1:1, TLCK rapidly and irreversibly inhibits the esterase activity of α-clostripain, since within 60 s of the reaction, the residual activity was less than 0.5% (fig.1). The extent of inhibition reaches the maximum value between pH 4–5; above this value it remains stable (fig.2). As the reaction is fast, it is not influenced by the known instability of TLCK under alkaline conditions [12]. In contrast to TLCK, even a 1000-fold molar excess of TPCK of PMSF over an extended period of time failed to inhibit the enzyme.

$HgCl_2$-treated clostripain did not react with even a 10-fold molar excess of TLCK and the subsequent removal of the mercury by dithiothreitol restored completely the original activity.

3.2. Stoichiometry of inhibition of α-clostripain by TLCK

Although the preparation of α-clostripain used for the inhibition experiments was homogeneous and the conditions for its maximum reductive activation were maintained, the inhibition was complete by less than a molar equivalent of TLCK calculated from the M_r of the proteinase (fig.1). The apparent molar concentrations of solutions of α-clostripain as determined by titration with TLCK were typically 70–80%. The corollary of

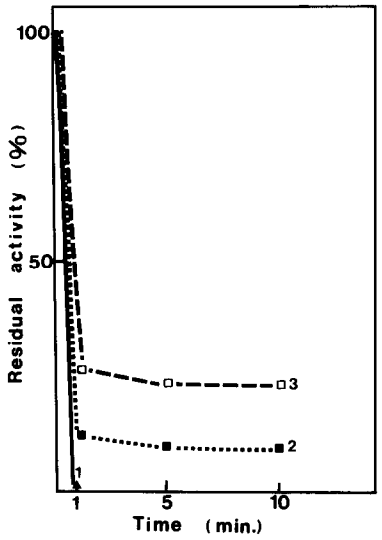

Fig.1. Effect of TLCK on the enzymatic activity of α-clostripain. α-Clostripain (spec. act. 440 units/mg) concentration: 3.6×10^{-6} M. Reactions were carried in 0.1 M phosphate buffer (pH 7.0) at 0°C without DTT. Curve 1, 4×10^{-6} M TLCK; curve 2, 2.8×10^{-6} M TLCK; curve 3, 2.0×10^{-6} M TLCK.

this is that the true specific activity of the fraction corresponding to the fully active enzyme in the rate assays is approx. 1.3-times the values we usually obtain with our preparations. For example, with our procedure of purification [4], we generally obtained an enzyme of apparent homogeneity and of spec. act. about 550–600 units/mg (on α-*N*-benzoyl-L-arginine ethyl ester) whereas the titration with TLCK gives a value of about 650–700 units/mg of completely active clostripain. The major reason why a preparation of fully activated α-clostripain cannot be obtained is its sensitivity to oxidation. Even after repeated removal of inactive clostripain by an ω-aminoalkylagarose column, partial oxidation always takes place. Consequently, apparent inhibition of α-clostripain by a less than equimolar quantity of TLCK is the result of the presence of catalytically inactive clostripain.

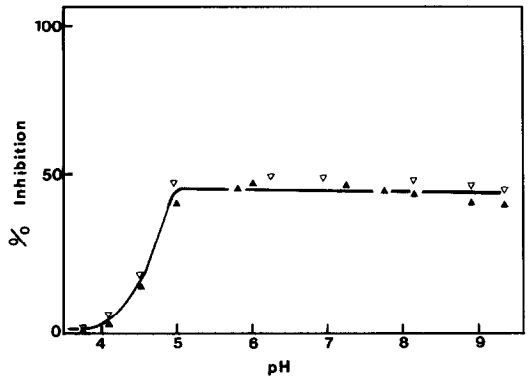

Fig.2. Effect of pH on the reactivity of TLCK with α-clostripain. Incubation was at 0°C. Concentration: 0.82×10^{-6} M, α-clostripain; 0.25×10^{-6} M, TLCK. Buffers: sodium acetate (pH 3.75, 4.10, 4.50 and 5.0); sodium phosphate (pH 5.80, 6.00, 6.25, 6.93 and 7.26); sodium borate (pH 7.77, 8.15, 8.90 and 9.35). The graph is the result of two experiments (▽,▲).

3.3. *Sulfhydryl group as the site of alkylation of α-clostripain by TLCK*

Simultaneously with the measurement of residual activity during TLCK treatment, the sulfhydryl content of the inactivated enzyme was determined by spectrophotometric titration with DTNB. A typical example is reported in fig.3 and illustrates the loss of titratable sulfhydryl goups concomitant with the loss in activity. In this experiment, a solution of 60% catalytically active α-clostripain had 0.8 titratable SH group. To this solution, TLCK at different molar ratios of inhibitor to enzyme was added and the residual titratable

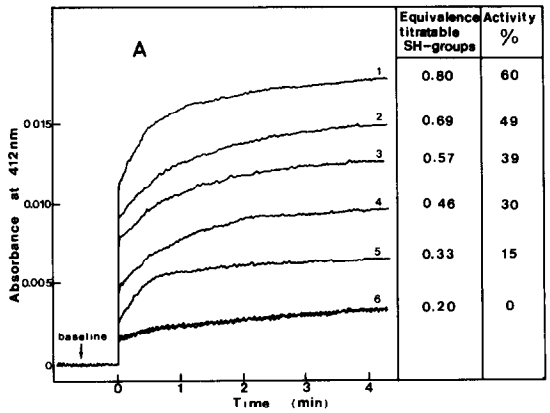

Fig.3. Typical recording at 412 nm (A) of titration of accessible SH group of α-clostripain by DTNB after reaction with TLCK. α-Clostripain was at 1.8×10^{-6} M and was 60% catalytically active. Curve 1 without TLCK; curve 2, 0.2×10^{-6} M TLCK; curve 3, 0.42×10^{-6} M TLCK; curve 4, 0.62×10^{-6} M TLCK; curve 5, 0.85×10^{-6} M TLCK; curve 6, 1.1×10^{-6} M TLCK and 4.0×10^{-6} M TLCK. The percentage of residual activity is reported.

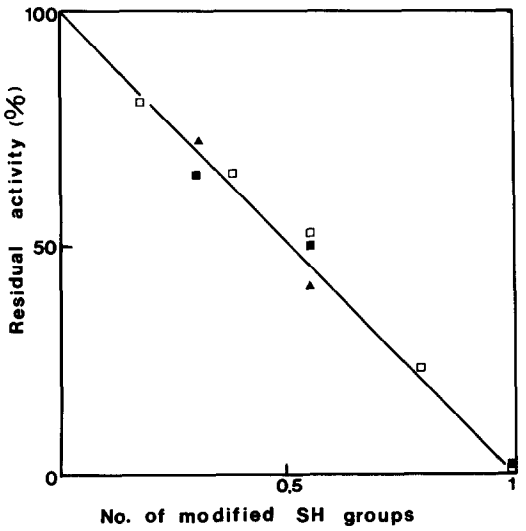

Fig.4. Relationship between the residual esterase activity of α-clostripain and the degree of substitution of the sulfhydryl group by TLCK at pH 7.0 and 0°C in the case of fully active enzyme (spec. act. 650–700 units/mg). The graph is the result of 3 experiments.

sulfhydryl groups were recorded. It was observed that even after complete loss of activity resulting from extended incubation with TLCK, about 20% of the sulfhydryl groups remained (fig.3, curve 6). The reaction of the sulfhydryl groups of α-clostripain with TLCK is dependent upon the active enzyme; the number of moles of sulfhydryl groups blocked per mole of enzyme by TLCK corresponded exactly to the fraction of active enzyme (normality) as determined by the assay with BAEE. Fig.4 illustrates the relation between the loss in activity and the number of labelled sulfhydryl groups in the case of fully active α-clostripain. A comparison of the amino acid analyses of the inhibited enzyme with the uninhibited control reveals that the only amino acid to have undergone a significant change was cysteine (8.2 residues found instead of 8.9) and that no decrease of histidine residues was observed (9.1 residues in both cases).

4. DISCUSSION

According to this study, TLCK inactivates α-clostripain by reacting with a sulfhydryl group. The results in support of this include: (i) loss of one titratable free sulfhydryl group simultaneous with loss of activity; (ii) loss of a cysteine residue as determined by amino acid analysis where there is no significant change in any of the other amino acids; (iii) $HgCl_2$-treated clostripain is not inactivated by TLCK; (iv) the stoichiometry of the reaction indicates that one TLCK reacts with one sulfhydryl group of α-clostripain which results in complete loss of activity. The conclusion that TLCK reacts with the sulfhydryl group of α-clostripain is in agreement with our preliminary study on the reaction of TLCK with clostripain [4] and with the hypothesis of authors in [5] on the mechanism of the reaction of TLCK with two other SH-proteinases, namely, papain [6] and ficin [7].

The reaction of α-clostripain with TLCK differs, however, in some aspects from that of trypsin or papain.

First, as in the case of papain [6], the rate of reaction of TLCK with clostripain is much faster than with trypsin. Under similar reaction conditions, 99% of the activity of α-clostripain is lost after 1 min incubation compared to 80% for trypsin after 1 h reaction. Second, TLCK reacts with the sulfhydryl group rather than with the imidazole group of a specific histidine as it does in the case of trypsin. On the basis of nucleophilicity alone, one can expect the attack of this chloromethyl ketone on a sulfhydryl group to be favored over that on an imidazole group. This was clearly illustrated by authors in [13] who succeeded in converting the hydroxyl group of the active serine of trypsin into a thiol group without a drastic change in the activity. In this thiol trypsin, TLCK reacted with the new thiol group with subsequent loss of activity without modification of histidine residues.

A third way in which the reactions differ is in the nature of the pH-profile of the reaction. For trypsin, the reaction with TLCK indicated dependence on a group with $pK_a = 6.25$ assignable to a histidine residue [12]. In the case of papain, the reaction with TPCK is dependent on a single prototropic group of pK 8.9 indicating the involvement of a sulfhydryl group [6]. For α-clostripain, taking in account the interactive active-center system characterized by two pK_a values of approx. 4 and 9 [14], the high reactivity with TLCK at a pH value around 4.5 may be attributed to the formation of the interactive system from a thiol, rendered nucleophilic, with the imidazole group of one histidine and which produces the nucleophilic ion pair ImH^+/S^-.

Another difference between papain and α-clos-

tripain is in the specificity of the reaction. Both TPCK and TLCK inhibit papain with the latter reacting at a faster rate. Papain is also rapidly inhibited by reaction with PMSF. In the case of α-clostripain only TLCK reacts. The rapid attack by TLCK on the active site would appear to contradict the restricted arginine specificity. In a previous study in our laboratory, an arginine cloromethyl ketone (p-NO_2-ZACK) was prepared in pure form and assayed with α-clostripain; it was evident that this reagent is extremely effective in the inactivation of both clostripain and trypsin [15]. During this study, however, this highly unstable reagent [16–18] was not available.

The present work gives direct evidence that TLCK inactivates α-clostripain stoichiometrically and that the reaction of the sulfhydryl group of clostripain with TLCK is dependent upon an active enzyme. After complete loss of activity on incubation with TLCK, some sulfhydryl groups remained. Furthermore, the number of moles of sulfhydryl groups reacted per mole of enzyme corresponds exactly to the fraction of active enzyme (normality) as determined by reaction with BAEE. Thus, one can determine the operational normality of a clostripain solution by use of TLCK and DTNB to titrate the sulfhydryl groups. In the absence of a 'burst assay' such as that with *p*-nitrophenyl-*p'*-guanidinobenzoate for trypsin [19], TLCK can be used as active-site directed inhibitor for α-clostripain.

Several other reagents have been proposed for the active-site cysteine residue in other proteinases: these include 2,2'-dipyridyl disulfide [20] and more recently the peptidyldiazomethanes [21]. The latter are unreactive with free cysteine but react irreversibly and rapidly in high dilution exclusively with thiol proteinases; they can be synthesized to satisfy the specificity of individual members of the thiol proteinase family. E64 [L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane], proposed as specific inhibitor for the cysteine proteinases, has no effect on α-clostripain [22].

In [8], we have isolated a tryptic peptide containing the sulfhydryl group of a cysteine residue labelled with iodo[1-^{14}C]acetic acid. This cysteine residue is located in position 41 of the heavy chain. The sequence of this peptide shows no homology with other cysteine proteinases such as papain, ficin, cathepsin B or streptococcal proteinase.

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