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Investigation of the inhibition mechanism of coumarins on chymotrypsin by mass spectrometry

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Abstract—6-Chloromethylcoumarin derivatives are known to express a marked inhibitory potency against serine proteases. However, their mechanism of inhibition remains unclear. In order to confirm the postulated mechanism, we use mass spectrometry. The shift mass obtained after inactivation by two compounds, which differ only by the nature of the leaving group (chloride or acetate) was in agreement with an alkylenzyme formation. With another compound devoid of a latent alkylating group, the shift mass obtained with the complex corresponds to an acylenzyme resulting from the interaction of the serine residue with the lactone carbonyl group. These results clearly demonstrate that the inhibition is not due to an attack of the exocyclic carbonyl group by the active serine but rather result from a nucleophilic attack on the intracyclic carbonyl group.

1. Introduction

Serine proteases play an important role in numerous physiological processes and are also involved in pathological states.[1,2] Among these proteases, human leukocyte elastase (HLE) could degrade various structural proteins including elastin, fibronectin and collagen.[3] Thus, an imbalance between HLE and the endogenous inhibitors could lead to tissue destruction. It has been suggested that HLE is a causative factor in emphysema,[4] chronic bronchitis,[5] acute respiratory distress syndrome,[6] cystic fibrosis[7] and arthritis.[8] Thrombosis is one of the leading single cause of morbidity and mortality in developed countries. The most widely studied target for antithrombotic intervention has been the serine protease thrombin which performs a dual role in thrombogenesis, including fibrin formation and platelet activation.[9,10] Previously, we have developed a new series of coumarins targeting serine protease especially α-chymotrypsin (α-CT),[11–13] HLE[12,14] and thrombin.[14] These compounds were designed as mechanism-based inhibitors. It was hypothesized that the lactone carbonyl group undergoes a nucleophilic attack by the active serine. This attack would be followed by the opening of the lactone resulting in the formation of an electrophilic quinone methide. This later compound could form a covalent bond with a nucleophilic residue within the enzyme recognition site. However, according to the structural feature of the inhibitors, several mechanical routes leading to inhibition are possible (Scheme 1).

Here, we will focus on the mechanism of inhibition. For this purpose, α-CT has been used as a model for studying the mode of action of these coumarins on proteolytic enzymes.

Mass spectrometry has been successfully employed for determination of the molecular mass of large intact proteins.[15] Moreover, this technique provides an excellent method for confirming the presence of covalently bonded groups in proteins.[16–18] Thus, we have used mass spectrometry in order to reveal the reactive pathway followed by the inhibitors. To reach this goal, three compounds have been tested. The enzyme–inhibitor complexes obtained with these inhibitors have been isolated and their mass compared to the native α-CT.

2. Results and discussion

The first postulated step corresponds to the formation of the acylenzyme **a** (pathway A, Scheme 1). In this step, the nucleophilic Ser-195 reacts with the lactone carbonyl group and forms an 'open' coumarin. In the case of 6-chloromethyl substituted coumarins, the ring opening of the lactone results in an increase of the leaving properties of the chlorine atom.[19] Therefore, after the leaving of the chloride ion, the electrophilic quinone methide could form a

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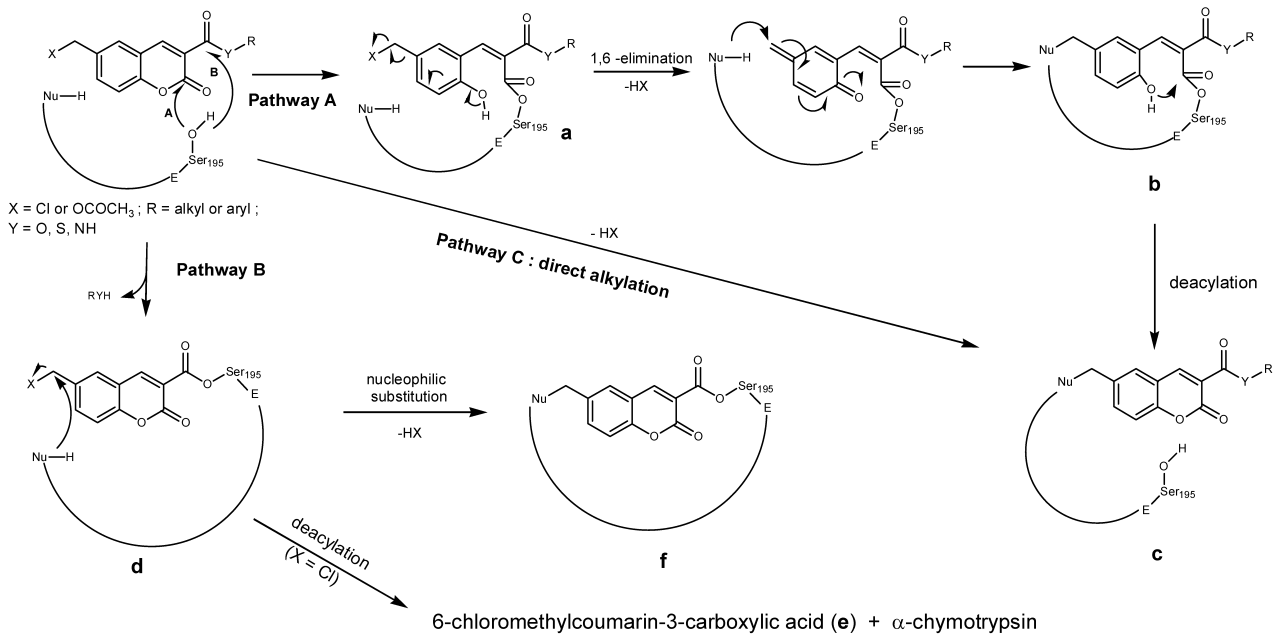

Scheme 1. Postulated mechanisms for inhibition of serine protease by coumarin derivatives.

covalent bond with a nucleophilic residue in the enzyme recognition site (**b**, Scheme 1). This latter could deacylate to form another alkylenzyme (**c**, Scheme 1). The alkylation of the enzyme was probably responsible for the irreversible inhibition of α-CT.

An alternative mechanism (pathway B) could be the reaction of the active Ser-195 with the exocyclic ester leading to the formation of the acylenzyme **d**. This complex could either slowly deacylate with reactivation of the enzyme and formation of 6-chlorometylcoumarin-3-carboxylic acid **e**, or a nucleophilic substitution could occur to form the alkylenzyme **f**. In such a case, an irreversible inactivation could be observed.

Finally, the inhibitors could act as 'affinity labels' (pathway C). In this case, the inactivation does not require a catalytic reaction to form a covalent bond with the enzyme. However, this reaction could be unspecific and the number of inactivator molecules covalently bounded per enzyme molecule should be superior to one.

According to these hypotheses, the α-CT inactivation could be the result of the formation of an acyl- or an alkyl-enzyme. These enzyme–inhibitor complexes differ by their mass. Therefore, mass spectrometry on the appropriate enzyme–inhibitor complex could be used to reveal the steps leading to inactivation.

It was previously reported that commercial samples of bovine α-CT contained several impurities from 25,230 to 25,692 Da.[20] In order to evaluate the purity of the starting enzyme material, we have used mass spectrometry. It appears that the Sigma α-CT gave two peaks of similar intensities at 25,235.9 and 25452.1 Da. With the Calbiochem and both Worthington samples, we observed a main peak at 25,235 Da which corresponds to the calculated value from the published amino acid sequence.[21]

Therefore, we have chosen the α-CT from Worthington origin (ref. CDI) for the inactivation studies. Furthermore, the purification step needed to isolate the enzyme–inhibitor complex was applied to the native α-CT. In this procedure, the enzyme was first concentrated by ultrafiltration and washed three times to remove salts. Then, before injection, the sample was fixed on a Zip Tip C18, washed with formic acid and eluted with methanol solution. This procedure led to a purer α-CT (Fig. 1).

Three compounds have been used to establish the different steps leading to enzyme inactivation (Fig. 2). Compound **1** was found to be one of the most powerful α-CT inactivator reported with a k_{inact}/K_I value over 700,000 $M^{-1}s^{-1}$. This product is characterized by a chloromethyl group in the 6-position which can alkylate the enzyme. Its partition ratio, which represents the average number of enzyme 'turnover per inactivation', is particularly good (r=1.8).[12] It is

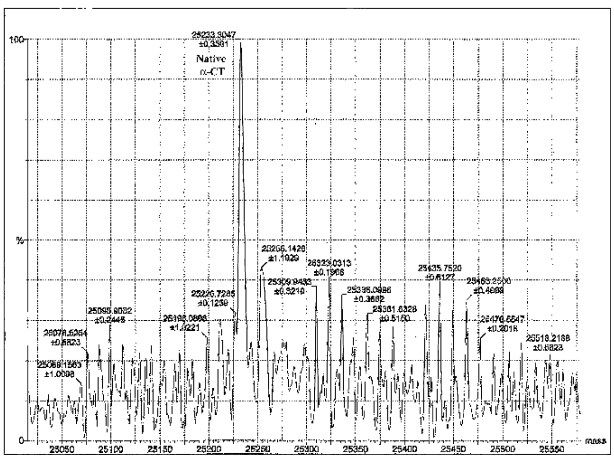

Figure 1. Mass spectrum of bovine α-CT, expected average mass 25,235 Da, after purification step.

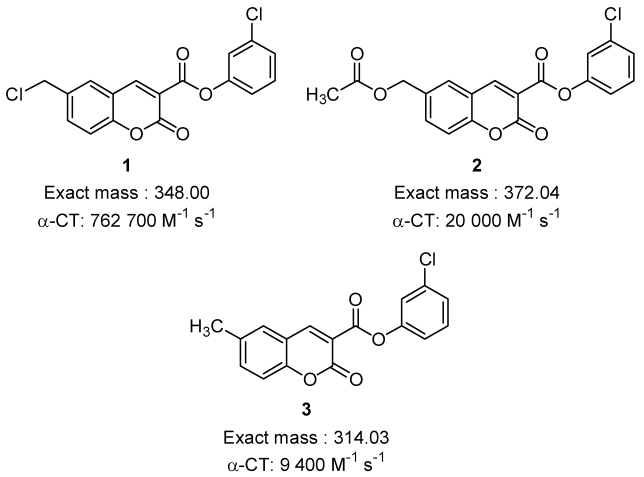

Figure 2. Exact mass and kinetic parameters (k_{inact}/K_I) for inactivation of α-CT by compounds **1**–**3**.

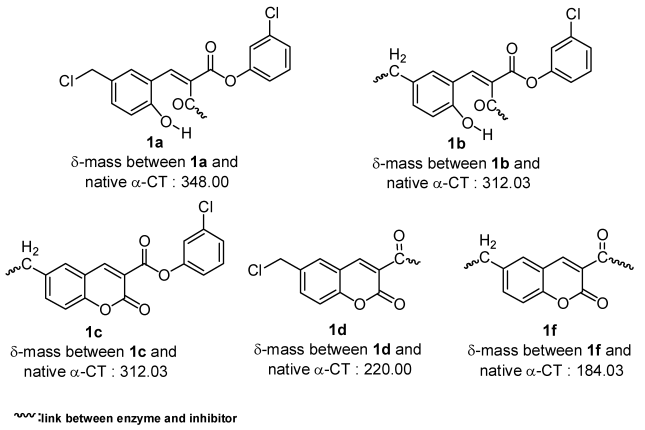

Figure 3. Δ-Mass between hypothetic complex and α-CT for compound **1**.

Table 1. Observed mass of native α-CT and enzyme–inhibitor complexes

Complex	Mass (Da)	Δ Mass between complex and native α-CT[a] (Da)
Native α-CT	25,235.0	0
α-CT-compound **1**	25,546.7	311.7
α-CT-compound **2**	25,546.5	311.5
α-CT-compound **3**	25,547.4	312.4

[a] Shift mass were calculated using the mixture between the complex and the native α-CT.

interesting to note that the ring opening increases the leaving properties of the chlorine atom by an 1,6-elimination from the benzyl intermediate. However, regarding the leaving properties of the chlorine atom, this compound could also act as an 'affinity label'.

For compound **2**, which was 40 fold less active, the chloromethyl group was replaced by an acetoxymethyl group. The α-CT inhibition with this latter was found to be irreversible with a poor partition ratio (r=80). With this molecule, the acetoxy moiety possesses a less nucleofugal leaving group [pk_a(acetate)=4.8 and pk_a(Cl)=−6.1)] and in other series of inactivators, it has been reported that the acetate moiety, as a leaving group, does not lead to irreversible inhibition.[22–24]

Surprisingly, compound **3**, devoid of a latent alkylating function, inactivate α-CT. In this case, the alkylation of the enzyme is impossible and the inactivation is probably due to the formation of a stable acylenzyme.

Thus, reactivation kinetic studies with compound **3** have been realized. For these studies, α-CT was completely inactivated. The excess inhibitor was removed by filtration and washing using a centricon (cut off 10,000). Then, the sample was treated with or without hydrazine (0.6 M final concentration) for 24 h at 25°C. The hydrolytic activity was determined on aliquots at 0, 4, 8 and 24 h after treatment. In these conditions, no reactivation (<1%) even after treatment with hydrazine was observed. This result indicates the irreversible nature of inhibition probably by forming an acyl-enzyme with the Ser-195 or the His-57. The stability of the enzyme–inhibitor complex in the presence of hydrazine was surprising since it was usually reported that hydrazine promotes the reactivation of simple acyl-enzymes.[25] Moreover, when the *m*-chlorophenyl group of the side chain in the 3-position of compound **3** was replaced by a phenyl group, the product was not an inhibitor but a substrate.[12] Thus, the presence of the chlorine atom prevents the reactivation of the enzyme.

The enzyme–inhibitor complexes were obtained by complete inactivation of α-CT. These complexes were, then, purified and concentrated by ultrafiltration on cellulose membrane and their mass were measured by direct infusion into the mass spectrometer.

Compound **1** forms a stable covalent complex with α-CT. The mass shift observed between the native α-CT and the enzyme–inhibitor complex (312 Da) is in agreement with the formation of a **1b** or **1c** type complex (Fig. 3, Table 1 and Scheme 1). This excludes pathway B as a route to inactivation (Scheme 1). Indeed this pathway would lead to a shift mass of 220 or 184 Da for **1d** and **1f**, respectively (Fig. 3). However, since the mass of complexes **1b** and **1c** are identical, mass spectrometry was not able to discriminate between the two complexes and to demonstrate the ring opening of the lactone.

A similar behavior occurs when the enzyme was incubated with compound **2** (Table 1). In this case, the acetate (exact mass of acetic acid 60.02 Da) has lower leaving properties. Nevertheless, the inhibition leads to the same complex as with compound **1** and the nature of the leaving group did not prevent the formation of the alkylenzyme. Thus, the release of AcOH is probably promoted by the 1,6-elimination from the benzyl intermediate formed after the lactone ring opening.

With compound **3**, the only possible covalent enzyme–inhibitor complex is the acylenzyme **a** (Scheme 1). The δ-mass obtained with the enzyme–inhibitor complex **3** was 312.4 Da (Table 1, Fig. 4) and corresponds to the theoretical Δ-mass for an acylenzyme **a** (Scheme 1, X=H and Δ=314.03 Da). This experiment clearly indicates that with compound devoid of a latent alkylating function, we

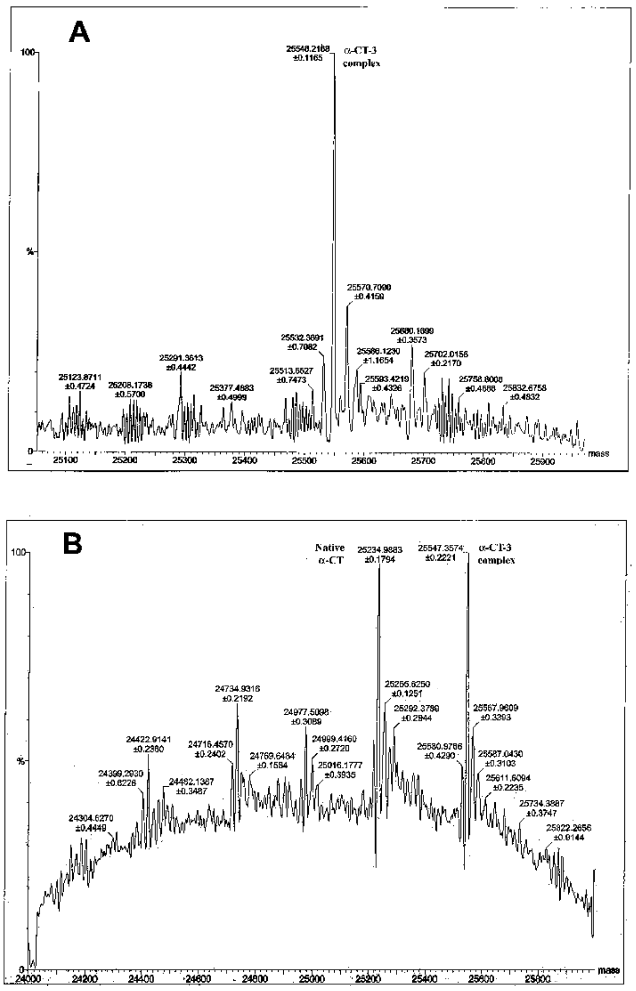

Figure 4. Mass spectra of α-CT-compound **3** complex (A), expected average mass 25,549 Da and a mixture (50/50) of native α-CT and α-CT-compound **3** complex (B).

probably obtained an acylenzyme formed by the reaction of the active serine with the carbonyl group of the lactone ring. The results obtained are in agreement with the recently published crystal structure of α-chymotrypsin complex with 7-hydroxycoumarin.[26] This structure reveals that the inhibition by 7-hydroxycoumarin leads to a planar cinnamate acylenzyme complex.

In conclusion, all these experiments are in agreement with the postulated mechanism in which the active serine attacks the lactone carbonyl group. This attack probably implies the formation of a cinnamate type acylenzyme complex. Whether the leaving group was an acetate or a chloride moiety, the acylenzyme evolves through an alkylenzyme complex suggesting that the alkylation properties of the inhibitors were promoted by the 1,6-elimination process from the benzyl intermediates. It is interesting to note that the acylenzyme complex formed with compound **3**, devoid of a leaving group, is stable whereas, the replacement of the *m*-chlorophenyl group by a phenyl one did not give an inhibitor but a substrate.[12] Thus, the presence of the chlorine atom on this phenyl ring strongly enhances the strength of interaction between the enzyme and the inhibitor.

3. Experimental

3.1. Materials

Bovine α-CT were purchased from Sigma (ref C4129), from Calbiochem (ref 230832), and from Worthington (ref CDI and CDS). Compounds **1**–**3** were synthesized according to previously published procedures.[11,12] In order to control inactivation, the α-CT was assayed spectrophotometrically using *p*-nitroanilide substrates (Sigma): succinyl-alanyl-alanyl-prolyl-phenylalanyl *p*-nitroanilide. The reactions were performed in 0.025 M sodium phosphate, 0.05 M NaCl and pH 7.5. The enzyme and substrate concentration were 1.1 μg/mL and 200 μM, respectively. Assays contained maximum 10% (v/v) acetonitrile and were run at 25°C in a lambda 20 bio Perkin–Elmer spectrophotometer equipped with a thermostated cell-holder.

3.2. Preparation of the inactivated samples

The enzyme–inhibitor complexes were prepared by portion-wise addition of inhibitors (1 mM in acetonitrile) to an enzyme solution (1 mg/mL for **1** and 0.01 mg/mL for **2** and **3**). This addition (60 to 100 μL) was performed until the enzyme was totally inactivated. Then, the complexes were concentrated by filtration on a centricon (cut off 10,000 Da) and washed three times in order to remove salts. The samples were analyzed by tandem mass spectrometry.

3.3. Hydrazine reactivation

The enzyme–inhibitor complex was prepared according to the procedure described in Section 3.2. In this case, the washing solution was the kinetic buffer. The sample obtained was then incubated in the presence or absence of hydrazine (final concentration 0.6 M) during 24 h at 25°C. Enzyme activity of aliquots was monitored and compared to the control. The chymotryptic activity of each group was expressed as a percent of control.

3.4. Mass spectrometry

Preliminary studies concerning various batches of commercially available α-CT have been performed by direct infusion of a α-CT solution (0.25 mg/mL) using a Howard Apparatus (USA) syringe pump. The mass spectrometer was a Platform II Micromass (Manchester).

Before injection, the samples were further purified by retention on a ZipTips C18, washed with 5% formic acid solution and eluted with 80% methanol solution into a nanospray capillary needle. Before use, the ZipTip was conditioned with a methanol solution and then 5% formic acid solution. Mass measurements were performed on a Micromass Q-Tof2 mass spectrometer (Manchester, UK) with the MaxLynx3.4 software.

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