Nature of papain products resulting from inactivation by a peptidyl *O*-acyl hydroxamate*

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Mass spectrometry has been used to provide insights into the mechanism of inhibition of cysteine proteases by a hydroxylamine derivative, CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph. An oxidized form of papain resulting from the incubation of the enzyme with the peptidyl hydroxamate in the absence of a reducing agent has been identified as a sulfinic acid. The presence of a covalent enzyme–inhibitor complex of molecular mass consistent with a sulfenamide adduct of papain could also be detected by this method. Implications on the mechanism of inactivation of cysteine proteases by peptidyl hydroxamates are discussed.

Cysteine protease; Papain; Peptidyl hydroxamate; Oxidation; Inhibition; Electrospray mass spectrometry

1. INTRODUCTION

Because of their role in many biological processes and their pathophysiological significance, cysteine proteases constitute prime targets for the design of specific inhibitors [1–3]. A number of compounds have been shown to irreversibly inhibit cysteine proteases, including chloro- and fluoromethyl ketones, acyloxymethyl ketones, diazomethyl ketones and peptidyl epoxides [1,4–6]. In recent years, a novel class of cysteine protease inhibitors has been developed based on the hydroxylamine function. Initially described as inhibitors of serine proteases [7], peptidyl hydroxamates have been shown to be much more effective inhibitors of cathepsin B and cysteine proteases in general [8,9]. In particular, *O*-mesitoyl derivatives of peptidyl hydroxamates have been shown to be very potent inhibitors of cathepsin B, with a second-order rate of inactivation comparable to those observed for chloromethyl ketone inhibitors [8]. It has been postulated that the inactivation mechanism could involve the formation of a tetrahedral intermediate similar to that found in the pathway for substrate hydrolysis and that this intermediate could break down to form either a sulfenamide or a thiolcarbamate [8]. More recently, the characterization by NMR of the complex formed between ^{13}C- and ^{15}N-labeled CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph and papain gives strong support for the sulfenamide as the covalent inactivation adduct of cysteine proteases by peptidyl hydroxamate [10]. In the same study, a competing inhibition mechanism has been uncovered in the absence of a reducing thiol. The inactive enzyme formed in this process is not the sulfenamide nor a thiolcarbamate. However, the fact that part of the activity of the enzyme can be recovered by addition of DTT suggests some type of oxidation product of papain.

In the present work, we have examined the enzyme products formed by reaction of the cysteine protease papain with the peptidyl-*O*-acyl hydroxamate CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph using the technique of electrospray mass spectrometry [11,12]. This technology is still in its infancy but is proving to be very valuable for studying biological systems. Since ionization occurs under mild conditions at atmospheric pressure, enzymes and their derivatives are not fragmented. This method therefore allows determination of the molecular masses of the papain products formed in the presence of the peptidyl hydroxamate.

2. MATERIALS AND METHODS

2.1. *Materials*

Papain was obtained from Sigma Chemical Co. and was purified and activated as described previously [13]. The inhibitor CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph was prepared as described previously [8]. The substrate CBZ-Phe-Arg-MCA was purchased from IAF Biochem International, Laval, Québec. Samples for mass spectrometric analysis were prepared by mixing enzyme and inhibitor in a 20% acetonitrile solution. No buffer was used for the mass spectrometry experiments since they interfere with the ionization process. Papain concentration was 40 μM and the pH was kept between 5.5 and 6.5. After adding the inhibitor, sufficient time was allowed for formation of the inactive papain products (determined by measuring residual

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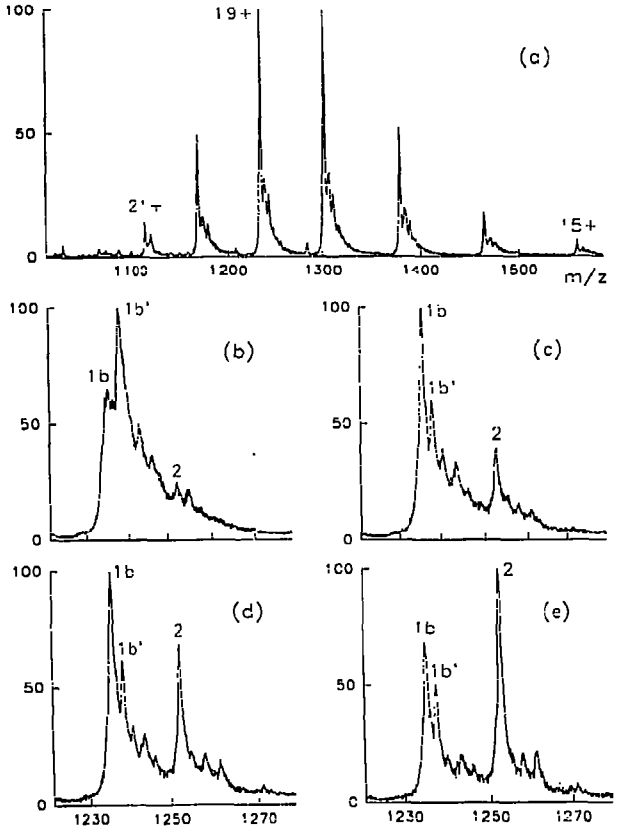

Fig. 1. (a) The electrospray mass spectrum of papain. The charge states ranging from 15+ to 21+ are shown. (b)–(d) Partial mass spectra of the enzyme from the 19+ charge-state region (m/z = 1220–1280) in presence of varying concentrations of the inhibitor CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph and in the absence of a reducing thiol. The ratio of inhibitor over enzyme is 1.0 (b), 4.9 (c), 10.0 (d) and 13.9 (e). Peak assignment corresponds to the numbered species in Fig. 2. Peaks 1b (23 454 Da) and 2 (23 777 Da) correspond to the oxidized and sulfenamide derivatives of papain respectively. The peak identified as 1b' is probably due to a sodium adduct of oxidized papain (23 500 Da, papain +32 + 2 Na). Other minor peaks correspond to sodium and sulfate adducts of papain, as described in the text.

activity against the substrate CBZ-Phe-Arg-MCA). Prior to injection in the mass spectrometer, the sample was acidified by adding acid up to a final concentration of 20%.

2.2. *Mass spectrometric analysis*

A triple quadrupole mass spectrometer (the API III LC/MS/MS system, Sciex, Thornhill, Ontario, Canada), the detailed description of which has appeared elsewhere [14–16], was used for all experiments. In brief, the mass spectrometer has an m/z (mass-to-charge) range of 0–2400 and is fitted with an ion-spray (pneumatically-assisted electrospray) interface [14]. Multiply charged protein ions were generated by spraying the sample solution through a stainless-steel capillary held at a high potential (+4–6 kV). The sample solution was delivered to the sprayer by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA) through a fused silica capillary of 100 μm ID. The liquid flow rate was set at 1.0 μl/min for sample introduction. The potential on the sampling orifice of the instrument was set at +35 V during calibration and was raised to +120 V for proteins to enhance ion signals. The instrument m/z scale was calibrated with the ammonium adduct ions of polypropylene glycol (PPG). The average molecular mass values of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions [15,17,18].

3. RESULTS AND DISCUSSION

Fig. 1a shows the electrospray mass spectrum obtained for papain. It consists of a series of peaks corresponding to different m/z ratios of the enzyme from which a value of 23 422 (± 1) is obtained for the average molecular mass of papain (calculated from the amino acid sequence = 23 422 Da). Upon adding the hydroxamate inhibitor, shifts in the m/z peaks are observed indicating the formation of papain products. This is illustrated for various ratios of inhibitor/enzyme in Fig. 1b–e, where only the region of m/z = 1220–1280 (corresponding to charge-state 19+) is shown. In the absence of a reducing thiol and at low inhibitor/enzyme ratios, the major peaks correspond to 23 454 (± 1) Da and 23 500 (± 2) Da (Fig. 1b). The former is attributed to an oxidized form of papain (papain + 2 oxygens) while the latter is believed to correspond to a sodium adduct of the oxidized enzyme (papain + 2 O + 2 Na^+). Sodium as well as sulfate adducts are often observed using this technique [19] and are present as minor peaks in all mass spectra obtained in this study. Gas phase collisional activation has reduced the abundance of these ion adducts, but is unable to remove them completely.

At higher ratios of inhibitor/enzyme, another significant peak appears in the mass spectrum (Fig. 1c–e). This is particularly evident in presence of 10.0 (Fig. 1d) and 13.9 (Fig. 1e) equivalents of the peptidyl hydroxamate. The molecular mass calculated for this peak is 23 777 (± 1) Da, which can be attributed to compound **2** in Fig. 2 (23 776 Da calculated for **2**). The mass spectrometry result alone cannot differentiate between the sulfenamide and thiolcarbamate products since they have the same molecular mass. However, in combination with previous NMR studies indicating the existence

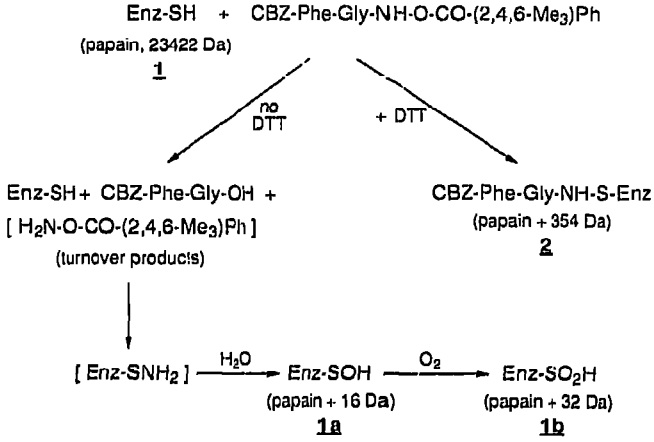

Fig. 2. Proposed pathways for reaction between papain and CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph.

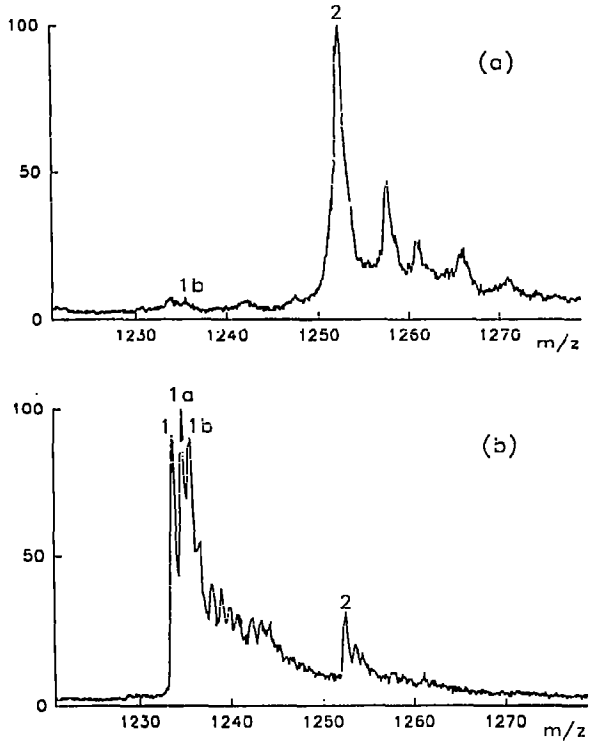

Fig. 3. Partial mass spectrum of papain (*m/z* region 1220–1280). (a) In presence of 13.9 equivalents of CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph and 2 mM DTT. Oxygen was removed from the enzyme and inhibitor solutions by bubbling nitrogen before starting the reaction. Peak assignments are as described in Fig. 1. (b) In the presence of 1.1 equivalent of CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph and in the absence of DTT and oxygen (removed by bubbling nitrogen in the solutions). Major peaks are observed corresponding to molecular masses of 23 422 Da (1, free papain), 23 439 Da (1a, sulfenic acid), 23 455 Da (1b, sulfinic acid) and 23 776 Da (2, sulfenamide).

of a sulfenamide type compound [10], the mass spectrometry result establishes convincingly that the species of 23 777 Da observed at higher inhibitor/enzyme ratios corresponds to compound **2**. It must be noted however that in the absence of a reducing thiol, the oxidized species is still a major product even when a considerable excess of inhibitor is used.

Fig. 3a shows the mass spectrum in the *m/z* 1220–1280 region of the inactivated form of papain (inhibitor/enzyme ratio = 13.9) obtained in solutions where oxygen was removed by bubbling nitrogen and in the presence of 2 mM DTT. Under these conditions, the oxidized papain is not observed and only one species of 23 777 Da corresponding to the enzyme–inhibitor adduct sulfenamide (**2**) is formed.

The existence of an oxidized form of papain was inferred from previous mechanistic studies of the inhibition of papain by peptidyl *O*-acyl hydroxamate, but the structure of this product was unknown [10]. It was previously shown that *O*-mesitoyl hydroxylamine, an expected turnover product, gives the same inhibitory effect [10], and it was proposed that this compound was the oxidant involved in this process. In fact, this reagent is known as an oxidant [20,21]. We propose that, based on the known chemistry of *O*-acyl hydroxylamines [20], the sulfenamide Enz-S-NH_2 is initially formed in this process, but is rapidly hydrolyzed to give the sulfenic acid Enz-S-OH (**1a** in Fig. 2) and NH_3 (previously detected as NH_4^+ by ^{15}N-NMR [10]). The sulfenic acid Enz-S-OH is expected to be a reactive species, readily reduced by thiol to give active enzyme [10], or air-oxidized further to give the more stable sulfinic acid Enz-SO_2H (**1b**) [22]. In the absence of DTT and oxygen, it should therefore be possible to detect the Enz-S-OH species. To test this possibility, the spectrum of a papain–inhibitor solution under conditions where the oxidative inhibition is predominant (i.e. inhibitor/enzyme ratio = 1.1 and in the absence of DTT) but where oxygen was removed by bubbling nitrogen, was monitored (Fig. 3b). Three major peaks are observed of 23 422 (± 1) Da (papain), 23 439 (± 1) Da (sulfenic acid **1a**) and 23 455 (± 1) Da (sulfinic acid **1b**). The sulfenic acid Enz-S-OH can indeed be observed under those conditions. Since nitrogen bubbling cannot completely remove all traces of O_2, the sulfinic acid **1b** is also present in solution. Free papain is observed in the sample of Fig. 3b due to incomplete inactivation under those conditions. A small amount of the sulfenamide adduct (**2**) is also observed. The molecular masses of the inactive forms of papain obtained in the absence of DTT (Figs. 1 and 3b) give strong support to the sulfenic and sulfinic acids **1a** and **1b** (Fig. 2) as the oxidized papain species and to the model of Fig. 2 for the mechanism of papain inactivation by CBZ-Phe-Gly-NH-O-CO-(2,4,6-Me_3)Ph. Such papain oxidation products have been proposed previously [23,24]. Our mass spectrometry results now provide evidence for papain sulfenic and sulfinic acids.

In conclusion, the interaction of papain with peptidyl *O*-acyl hydroxamates is a complex process involving two competing reaction pathways [10], which affords different inactive enzyme products depending on the conditions. Evidence from a combination of NMR studies [10] and electrospray mass spectrometry strongly indicates the formation of a novel sulfenamide enzyme-inhibitor adduct **2** in the presence of reducing thiol. Mass spectrometry studies have provided evidence supporting the formation of papain sulfenic and sulfinic acids (**1a** and **1b**) in the absence of thiol, apparently formed via the action of the putative turnover product *O*-mesitoyl hydroxylamine [10]. Electrospray mass spectrometry is clearly an invaluable tool for the characterization of enzyme derivatives obtained from enzyme-inhibitor interactions.

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