The high stability of cruzipain against pH-induced inactivation is not dependent on its C-terminal domain

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Abstract Unlike mammalian lysosomal cysteine proteases, the trypanosomal cysteine protease cruzipain contains a 130-amino acid residue C-terminal domain, in addition to the catalytic domain, and it is stable at neutral pH. The endogenous (with C-terminal domain) and recombinant (without C-terminal domain) cruzipains exhibit similar stabilities at both acid ($k_{inac} = 3.1 \times 10^{-3}$ s^{-1} and 4.4×10^{-3} s^{-1} at pH 2.75 for endogenous and recombinant cruzipain, respectively) and alkaline pH ($k_{inac} = 3.0 \times 10^{-3}$ s^{-1} and 3.7×10^{-3} s^{-1} at pH 9.15 for endogenous and recombinant cruzipain, respectively). The pH-induced inactivation, which is a highly pH dependent first order process, is irreversible and accompanied by significant changes of secondary and tertiary structure as revealed by circular dichroism measurements. The different stability of cruzipain as compared to related proteases, is therefore due mainly to the different number, nature and distribution of charged residues within the catalytic domain and not due to addition of the C-terminal domain.

Key words: Cruzipain; Lysosomal cysteine protease; Cathepsin; pH-stability; *Trypanosoma cruzi*

1. Introduction

The parasite-derived proteases have received considerable attention during the last decade due to their participation in the pathogenesis and survival of the parasite [1,2]. The protozoan parasite *Trypanosoma cruzi* is the causative agent of the American trypanosomiasis, Chagas' disease, which is transmitted to a mammalian host from the bite of an infected blood-sucking triatomine bug (reviewed in [3]). Cruzipain [4], also known as GP57/51 [5] or cruzain [6], is the major cysteine protease of *T. cruzi* and belongs to the papain family. The enzyme may participate in parasite nutrition [7], penetration into the host cells [8], pathogenesis and replication of the parasite [9], activation of plasma prekallikrein in the host [10] and protein breakdown [11].

The cruzipain gene encodes a protein containing a prepro-region, a catalytic domain and a C-terminal extension [6,12]. Evidence was presented that the C-terminal domain may be autocatalytically removed [13]. In recombinant cruzipain, expressed in *Escherichia coli* as a fusion polypeptide, autoactivation was accompanied by proteolytic removal of the pro-domain and the C-terminal extension [6,14].

Only cysteine proteinases from Trypanosomatids share an unusual 100–130-amino acids long C-terminal domain [6,15,16]. This domain is not required for protein folding, enzyme catalysis [6] and trafficking [17]. Its precise role is unknown, but it may significantly enhance the catalytic efficiency of these proteinases [10] and is also known to be the major immunogenic part of cruzipain [18]. Cruzipain is substantially more stable and active at neutral pH than the related cathepsins B and L [19–23], although the three-dimensional structures of the catalytic parts of the three enzymes are highly similar [24–27], suggesting that the C-terminal domain may play a role in cruzipain stability, as well as in parasite survival under the physiological conditions of the host. Therefore, the aim of this study was to investigate the role of pH in the regulation of cruzipain activity, as well as the possible contribution of the C-terminal domain to enzyme stability. We demonstrate here that the higher stability of cruzipain at neutral pH compared to related proteases is mainly due to different charged residues within the catalytic domain and not due to addition of the C-terminal domain.

2. Materials and methods

2.1. Materials

The substrate Z–Phe–Arg–MCA was purchased from the Peptide Research Institute (Japan). *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and glycine were provided by Serva (Germany). Dithiothreitol and ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma (USA). Dimethyl sulfoxide was from Merck (Germany). All other chemicals were of analytical grade. Stock solution of the substrate was prepared in dimethyl sulfoxide.

Cruzipain was isolated from *T. cruzi* epimastigotes, Tulahuén strain, Tul 2 stock [19]. Recombinant cruzipain, lacking the C-terminal extension, was expressed in *E. coli* as described earlier [14]. Protein concentration was determined from absorbance measurements by the method of Pace et al. [28].

The following buffers were used in kinetic experiments: 50 mM

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); k_{obs}, observed inactivation rate constant; k_{inac}, true inactivation rate constant; MCA, 4-methyl-7-coumarylamide; Z-, benzyloxycarbonyl

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citrate from pH 2.25 to 3.6, 50 mM sodium acetate from pH 3.7 to 5.6, 100 mM phosphate from pH 5.7 to 7.5, 50 mM HEPES from pH 7.6 to 8.5, 50 mM glycine from pH 8.6 to 10.3 and 50 mM boric acid/NaOH at pH 12.0. The assay buffer was 100 mM phosphate buffer, pH 7.0. All buffers contained 1 mM EDTA and 0.1 M NaCl.

2.2. Inactivation kinetics

The kinetics of inactivation of endogenous and recombinant cruzipain was studied at acidic and alkaline pH by continuous and discontinuous methods essentially as described previously [20,29]. All kinetic experiments were performed at 37°C, if not otherwise stated. Prior to the experiments, cruzipain was activated with 2 mM dithiothreitol as described [19,20]. The concentration of dimethyl sulfoxide was adjusted to 3% (v/v), unless otherwise stated, and substrate consumption was less than 5%.

2.3. Reversibility of inactivation

The reversibility of the pH-induced inactivation of cruzipain was studied at 37, 25 or 5°C as follows. 5 μl of cruzipain (0.1 μM) were added to 95 μl of prewarmed buffer (37°C) at pH 2.25 or 10.0 and incubated at the same temperature for different times. 20 μl were neutralized with 180 μl of thermostated assay buffer (pH 7.0; 37°C, 25°C or 5°C) and 5 μl aliquots were taken at appropriate time intervals to measure the residual activity, as described [21]. In the control experiments the enzyme was diluted into the assay buffer.

2.4. Circular dichroism

Cruzipain was inactivated at pH 2.3 and 10.3. The far-UV and near-UV circular dichroism spectra of active and inactivated cruzipain samples were recorded at 25°C with an AVIV 62 DS spectropolarimeter (Lakewood, NJ, USA) as described [30].

3. Results

3.1. Inactivation kinetics at acidic pH

The exposure of endogenous and recombinant cruzipain to pH 2.75 in the presence of substrate resulted in an exponential decrease of activity monitored by the release of the fluorescent MCA product (Fig. 1). The progress curves recorded could be best described by the simple first-order rate equation [20]. The substrate consumption was below 5% indicating that the decrease of activity was a result of a pH-induced inactivation. Also in the absence of substrate, the cruzipain activity at pH 2.75, monitored by a discontinuous method, progressively decreased in agreement with a single exponential decay.

Inactivation of both cruzipain forms was studied at pH 2.75 as a function of enzyme concentration by the continuous method. In the range covered (0.5–5 nM) the observed inactivation rate constant (k_{obs}) was independent of enzyme concentration ($k_{obs} = 19.7 \pm 0.2 \times 10^{-4}$ s^{-1} and $18.6 \pm 0.1 \times 10^{-4}$ s^{-1} for endogenous and recombinant cruzipain, respectively), indicating that the reaction is first order. In addition, this independence of enzyme concentration indicated that autolysis was not responsible for the enzyme inactivation. However, under the same conditions, the k_{obs} of both enzymes decreased with increasing substrate concentration (2.5–102.5 μM Z–Phe–Arg–MCA; Fig. 2), indicating that the enzyme–substrate complex is substantially more stable than the enzyme alone. The substrate dependence of the k_{obs} values could be described by the following competitive mechanism [20].

$$\begin{array}{ccc} & \overset{K_m}{} & \\ E + S & \rightleftharpoons & ES \longrightarrow E + P \\ \downarrow k_{inact} & & \\ E^* & & \end{array}$$

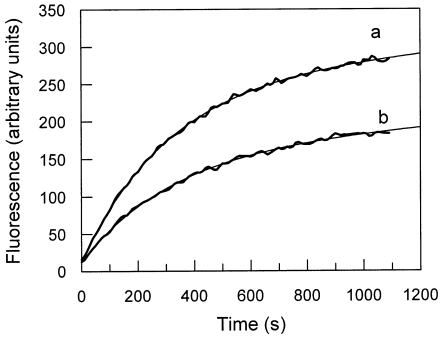

Fig. 1. Inactivation of endogenous and recombinant cruzipain at pH 2.75 and 37°C in the presence of substrate. The experimental data were fitted using the equation $F = F_\infty\ (1 - e^{-k_{obs} t}) \pm F_o$ where F and F_∞ represent the fluorescence measured at time t or infinite time and F_o stands for the background fluorescence. The values $k_{obs} = 2.70 \pm 0.02 \times 10^{-3}$ s^{-1} for recombinant cruzipain (a) and $k_{obs} = 2.45 \pm 0.04 \times 10^{-3}$ s^{-1} for endogenous cruzipain (b) were obtained. The enzyme and substrate concentrations were 0.5 nM and 25 μM, respectively.

where E and E* represent active and inactive enzyme, respectively, S stands for substrate and P for product. As can be seen, this model could be adequately fitted to the experimental data (Fig. 2), giving the best estimates of $3.1 \pm 0.1 \times 10^{-3}$ s^{-1} and $4.2 \pm 0.2 \times 10^{-3}$ s^{-1} for the substrate-independent true inactivation rate constant k_{inac}, and of 35 ± 4 μM and 25 ± 4 μM for the K_m for endogenous and recombinant cruzipain, respectively. At the same pH, the values of k_{inac}, determined by the discontinuous method, were almost identical (3.1×10^{-3} s^{-1} and 4.4×10^{-3} s^{-1} for endogenous and recombinant cruzipain, respectively).

3.2. Effect of pH and temperature on the inactivation rate constant

The inactivation process was studied at acidic pH (2.25–4.0) and alkaline pH (7.5–10.0). The rate of inactivation increased ~7300-fold from pH 4.0 to 2.25 and ~600-fold from pH 7.5 to 10.0 (Table 1). In the rate limiting step of the inactivation process 2.3 ± 0.2 protons were adsorbed at acidic pH and 0.95 ± 0.02 proton was desorbed at alkaline pH, as calculated from the slopes of the plot of log k_{inac} vs. pH [31] for endogenous cruzipain (not shown). The lack of a plateau in this plot

Table 1
Rates of inactivation of endogenous and recombinant cruzipain under acidic and alkaline conditions

pH	Endogenous cruzipain $10^3 \times k_{inac}$ (s^{-1})	Recombinant cruzipain $10^3 \times k_{inac}$ (s^{-1})
2.15	24.7 ± 0.1	50.6 ± 0.1
2.50	7.2 ± 0.2	22.1 ± 0.1
2.75	3.1 ± 0.1	4.4 ± 0.3
3.00	0.45 ± 0.03	N.D.
3.25	0.13 ± 0.01	N.D.
3.50	0.028 ± 0.002	N.D.
4.00	0.0032 ± 0.0003	N.D.
7.50	0.028 ± 0.001	N.D.
8.00	0.18 ± 0.01	N.D.
8.50	0.53 ± 0.03	N.D.
9.15	3.0 ± 0.1	3.7 ± 0.4
9.50	3.1 ± 0.5	7.1 ± 0.4
10.00	16.8 ± 1.1	N.D.

The best estimates for k_{inac} are given with their standard errors. Experimental conditions are given in Section 2.

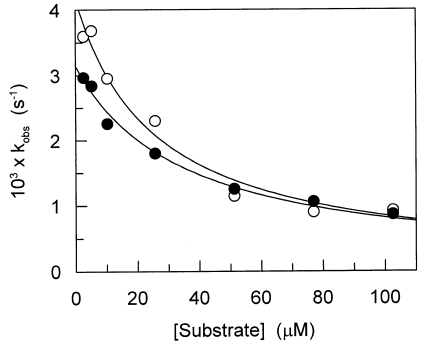

Fig. 2. Effect of substrate concentration on k_{obs}, measured in the presence of substrate, at pH 2.75 and 37°C. The experimental data were fitted using the equation $k_{obs} = k_{inac}/(1+[S]/K_m)$ [20], and the best estimates of $k_{inac} = 3.1 \pm 0.1 \times 10^{-3}$ s^{-1} and $K_m = 35 \pm 4$ μM for endogenous cruzipain (●) and $k_{inac} = 4.2 \pm 0.2 \times 10^{-3}$ s^{-1} and $K_m = 25 \pm 4$ μM for recombinant cruzipain (○), respectively, were obtained.

indicated that the pK_a values of the ionizing groups involved in the rate limiting step of the inactivation process were between 4.0 and 7.5. The inactivation rate constants of recombinant cruzipain, determined in the absence of substrate, were up to three-fold higher than those for endogenous cruzipain (Table 1) showing a small stabilizing effect of the C-terminal domain.

The temperature-dependence of the inactivation rate constant was studied in the range 5–37°C at pH 2.25 and 10.0. The rate of inactivation of endogenous cruzipain in this range increased ~870-fold at pH 2.25 and ~545-fold at pH 10.0.

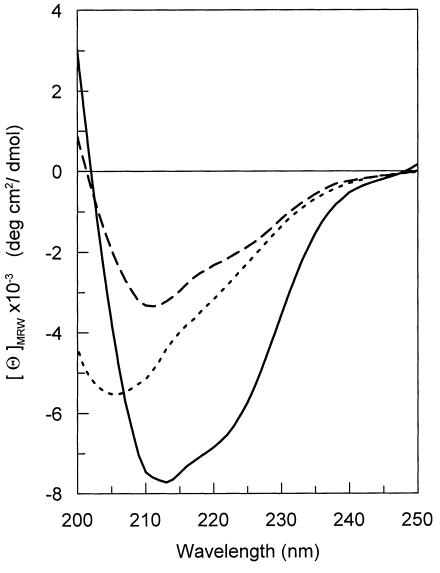

Fig. 3. Far-UV CD spectra of endogenous cruzipain at acidic, neutral and alkaline pH. Experimental conditions were as described in Section 2. The spectra were obtained at pH 2.3 (dashed), pH 6.5 (solid) and pH 10.3 (dotted). The enzyme concentration was 1 μM. Calculations by CONTIN method [32], gave the following fractions of secondary structural elements:

pH	α-helix	β-structure	Remainder
2.3	0.09 ± 0.01	0.50 ± 0.01	0.42 ± 0.01
6.5	0.24 ± 0.01	0.43 ± 0.01	0.33 ± 0.01
10.3	0.12 ± 0.01	0.40 ± 0.01	0.48 ± 0.01

From the slope of the Arrhenius plot (not shown), activation energies, of 177 ± 7 kJ mol^{-1} and 172 ± 10 kJ mol^{-1} at pH 2.25 and pH 10.0, respectively, were calculated.

3.3. Circular dichroism

The inactivation of cruzipain at pH 2.3 and pH 10.3 was accompanied by substantial conformational changes, as shown by far-UV circular dichroism spectra (Fig. 3). The far-UV CD spectrum of native cruzipain at pH 6.5 is characterized by a minimum at 213 nm, whereas a blue shift to 211 nm at pH 2.3 and to 206 nm at pH 10.3 was observed (Fig. 3). At both extreme pH values, a 50–60% decrease in α-helix content occurred, as judged by the CONTIN method [32]. Similarly, substantial changes were observed in the near-UV CD spectra (not shown).

3.4. Reversibility and light scattering studies

The reversibility of cruzipain inactivation at pH 2.25 and pH 10.0 was studied by exposing the enzyme to the inactivation buffer at 37°C for different times and then rapidly neutralizing the solution. No regain of activity was detected from the completely inactivated enzyme, and the increase in catalytic activity after shorter inactivation times never exceeded 10%. Since very low catalytic activity was recovered at 37°C, the renaturation studies were also followed at 25°C and 5°C. Nevertheless, the regain of activity was found to be similarly low at these temperatures, suggesting that the pH changes induced irreversible structural changes.

4. Discussion

In order to investigate the role of the C-terminal domain in cruzipain pH-stability, inactivation experiments were performed with endogenous cruzipain and a recombinant form of the enzyme lacking the C-terminal domain. The pH-induced inactivation of both cruzipain variants was shown to be a first order process. The rate of inactivation of both variants decreased with increasing substrate concentration, indicating that the enzyme–substrate complex is substantially more stable than the enzyme itself. However, the K_m value was ~30-fold greater at pH 2.75 (K_m values of 35 ± 4 μM and 25 ± 4 μM for endogenous and recombinant cruzipain, respectively) than at pH 5.8 (K_m value of 0.96 μM; [14]), indicating that the substrate-protective effect is stronger closer to neutral pH. The very good agreement of the k_{inac} in the presence and absence of substrate indicated that the results are independent of the method used. The inactivation rate constants for both cruzipain variants were similar in the whole pH range studied (Table 1), indicating that the C-terminal domain of cruzipain has very little effect on the stability of the enzyme. However, a strong dependence of k_{inac} on pH indicated on importance of ionic interactions for the stability of the enzyme.

The inactivation process was accompanied by significant changes in secondary and tertiary structure, as revealed by circular dichroism measurements. These structural changes are in agreement with the high activation energy for the inactivation of cruzipain under both pH conditions (~175 kJ/mol) and with that for the inactivation of other related enzymes like cathepsins B [21] and L [20,29]. The structural changes of endogenous cruzipain as well as the loss of cruzipain activity, were irreversible due to enzyme aggregation. These observations indicate that the C-terminal domain also

is not important for the reversibility of pH-induced inactivation.

Inactivation of both cruzipain variants is consistent with the mechanism, where inactivation starts in the active site region with disruption of the Cys–His active site ion pair and is followed by domain separation and further unfolding of the enzyme [21]. The different pH-stabilities of papain-like cysteine proteinases are probably a result of different number, nature and distribution of charged residues.

The high stability of cruzipain at neutral pH may have an important role in the physiological survival of the parasite. It is known that the survival of *T. cruzi* after human infection depends on the developmental stage [33]. Moreover, the presence of active cruzipain on the cell surface of amastigotes and trypomastigotes [34] might be relevant to the escape of the parasite from the phagosome and replication in the cytoplasm. The C-terminal domain may be involved in parasite survival as the major immunogenic part of the cruzipain molecule [18]. After human infection, the antibodies formed are probably directed to the C-terminal domain [5] and as a consequence, the catalytic part of cruzipain might not be inhibited by circulating protein inhibitors, due to the steric hindrance. The resulting immunocomplexes are as enzymatically active as the free enzyme. The structural bifunctionality of cruzipain, consisting of a highly stable catalytic domain and a highly antigenic C-terminal domain [18], may be essential for the parasite survival after host invasion. These studies contribute to the understanding of the higher stability of cruzipain compared to related mammalian lysosomal cysteine proteases, although the function of the C-terminal domain remains as yet unknown.

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