Inhibition of cruzipain, the major cysteine proteinase of the protozoan parasite, *Trypanosoma cruzi*, by proteinase inhibitors of the cystatin superfamily

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Abstract Cruzipain, the major cysteine proteinase from *Trypanosoma cruzi* epimastigotes, purified to a sequentially pure form, exists in multiple forms with pI values between 3.7 and 5.1, and an apparent molecular mass of 41 kDa. The enzyme is stable between pH 4.5–9.5. Cruzipain was found to be rapidly and tightly inhibited by various protein inhibitors of the cystatin superfamily (k_{ass} = 1.7–79 × 10^6 $M^{-1}s^{-1}$, K_d = 1.4–72 pM). These results suggest a possible defensive role for the host's cystatins after parasite infection, and may be of use for the design of new therapeutic drugs.

Key words: Trypanosoma cruzi; Cruzipain; Cysteine proteinase; Cystatin; Inhibition; Kinetics

1. Introduction

Cysteine proteinases comprise a group of enzymes which share extensive sequence homology and a common catalytic mechanism [1]. They are found in bacteria, protozoa, plants and animals. The protozoan parasite *Trypanosoma cruzi* is the causative agent of the American trypanosomiasis, Chagas' disease, which affects more than 24 million people in Latin America. It contains a major cysteine proteinase, cruzipain [2] that is a high mannose-type glycoprotein [3] present in the different developmental forms of the parasite [4] and is located in the lysosomes [5]. The enzyme is an immunodominant antigen, recognised in human sera from patients with chronic Chagas disease [6]. Cruzipain may be involved in the defense mechanism of the parasite against the host immune response, both by hydrolyzing the Fc moiety of antibodies [7] and by participating in the penetration of the trypomastigote into the mammalian cell [8]. Recent studies with inhibitors have shown its relevance in the differentiation steps of the parasite's life cycle [9].

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Abbreviations: -AMC, 4-methyl-7-coumarylamide; EDTA, ethylenediaminetetraacetic acid; E-64, 1-[L-*N*-(*trans*-epoxysuccinyl)leucyl]amino-4-guanidino butane; Ep-475, L-*trans*-epoxysuccinylleucylamido-(3-guanidino)butane; FPLC, fast protein liquid chromatography; M_r, relative molecular weight; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; SDS, sodium dodecyl sulphate; Z-, benzyloxycarbonyl.

Cruzipain has been purified from epimastigotes of *T. cruzi* and characterized [10–12]. It is inhibited by E-64, a strong irreversible inhibitor of cysteine proteinases [11]. Cruzipain is composed of two domains: a catalytic domain homologous to papain-like proteinases [13] and a domain which represents a C-terminal extension to the mature enzyme [14]. This C-terminal domain can be removed autocatalytically from the catalytic domain [15].

Cruzipain is encoded by a large number of tandemly arranged genes containing repeated units. The genes code for the preproenzyme form, complete with C-terminal extension [3,16,17]. Very recently, recombinant cruzipain without this C-terminal domain (also known as cruzain) was crystallized and its structure in complex with Z-Phe-Ala-fluoromethyl ketone determined [18].

Although the cystatins are well known to be strong competitive inhibitors of cysteine proteinases [19], there are no data available concerning their action on cruzipain.

In this report we present evidence that cystatins are also very potent inhibitors of cruzipain, thus indicating their possible defense role in the host organism. They may serve as promising starting points for the design of non-covalently bound reversible inhibitors as new antiparasite drugs.

2. Materials and methods

2.1. Inhibitors

Human stefin A [20], recombinant human stefin A [21], recombinant human stefin B [22], chicken cystatin [23], recombinant human cystatin C [24] and human low molecular weight kininogen [25] were purified by published procedures.

The proportion of active inhibitor in each preparation was determined by titrations against active-site titrated papain (EC 3.4.22.2, 2 × crystallized, Sigma), monitored by loss of enzyme activity [26], or by fluorescence titrations with purified papain [23]. The active concentration of kininogen was determined using a 2:1 enzyme to inhibitor binding stoichiometry (B. Turk, unpublished results).

2.2. Enzyme purification

Cruzipain was isolated from a cell-free extract of *T. cruzi* epimastigotes as previously described [10]. The material from the Mono Q chromatography step was dialysed against 0.1 M acetate buffer pH 5.5, containing 0.3 M NaCl and 1 mM EDTA, and applied to a Superdex 75 HR 10/30 column (Pharmacia, Sweden) equilibrated with the same buffer. The pooled active fractions were concentrated and stored at −20°C until used. The active concentration of cruzipain was determined by active site titration with Ep-475 [26].

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2.3. Analytical methods

Protein concentration was determined spectrophotometrically at 280 nm with the use of published [21,23] or calculated absorbance coefficients and molecular masses obtained from amino acid sequences [27].

SDS-PAGE was performed under non-denaturing conditions on 8–25% PhastSystem gradient electrophoresis gels (Pharmacia, Sweden). Analytical isoelectric focussing was done on PhastSystem Gel IEF 3–9 slabs (Pharmacia, Sweden), following the manufacturer's instructions. Proteins were stained with Coomassie brilliant blue (Pharmacia, Sweden).

N-terminal sequence analysis was performed on an Applied Biosystems 475A liquid-pulse sequencer, connected to a 120A PTH Analyzer from the same manufacturer.

2.4. Enzyme assay

Cruzipain activity was assayed using 10 μM Z-Phe-Arg-MCA (Bachem, Switzerland) in 0.1 M phosphate buffer pH 6.5, containing 1 mM EDTA and 2 mM dithiothreitol [28].

After 10 min incubation, the reaction was stopped with iodoacetic acid and the fluorescence of the released amidomethylcoumarin measured at excitation and emission wavelengths of 370 and 460 nm respectively; using a Perkin Elmer Luminescence-spectrometer LS 30 (USA). Measurements to determine the kinetic constants were made using a Perkin Elmer Luminescence-spectrometer LS 50 (USA).

2.5. Determination of pH optimum and pH stability

These properties were measured in buffers, made from 0.1 M citric acid and 0.2 M $Na_2HPO_4 \cdot H_2O$ (pH 3.0–8.0) and 0.05 M Tris/HCl (pH 8.0–11.0).

The activity of cruzipain (0.5 nM final concentration) was assayed as described in 2.4 at 0.5 pH unit intervals.

The pH stability was determined by incubation of 10 μl of enzyme and 90 μl of buffers of the given pH for 30 min at 37°C. Activity was then assayed at the optimum pH, 6.5.

2.6. Determination of K_m

The initial velocities of hydrolysis of different concentrations of Z-Phe-Arg-MCA by preactivated cruzipain were measured as in section 2.4. The substrate was dissolved in 0.1 M phosphate buffer pH 6.5, containing 1.5 mM EDTA. K_m and V_{max} values were obtained by non-linear regression analysis.

2.7. Kinetics and equilibrium of interaction with inhibitors

Association rate constants and inhibition constants for the reaction of cruzipain with recombinant stefin A and chicken cystatin were measured in 0.05 M Tris/HCl pH 7.4, containing 0.1 M NaCl and 100 μM EDTA, as described [21]. The kinetics of dissociation of the complex of cruzipain with chicken cystatin, form 2, were evaluated in the same buffer by trapping the enzyme dissociated from the complex with an excess of chicken cystatin, form 1 [29]. Association and dissociation rate constants for the interaction of cruzipain with stefin A, recombinant stefin B, recombinant cystatin C and kininogen were measured in the same buffer by methods described previously [26]. The measurements of kinetic and equilibrium constants were made by continuous rate assays in the presence of 10 μM Z-Phe-Arg-AMC, with at least a ten-fold molar excess of inhibitor over enzyme. Fluorescence was measured as in section 2.4. or in a DX 17MV stopped-flow instrument (Applied Photophysics, UK), with excitation at 360 nm and emission observed through a cut-off filter with 50% transmission at 400 nm [30]. Cruzipain was activated with 1–2 mM dithiothreitol for 5 min before each measurement. Less than 5% substrate was hydrolysed in all experiments.

3. Results and discussion

3.1. Enzyme purification and properties

The cysteine proteinase cruzipain was purified from *T. cruzi* epimastigotes by a published procedure up to the Mono Q chromatography step [10], with an additional gel chromatography step on Superdex 75 which separated cruzipain from some low molecular mass proteins. After this final step, which improves on the Superose 12 gel filtration previously used [10], the enzyme appeared pure by N-terminal sequence analysis. The first 10 amino acid residues corresponded to the published N-terminal sequence of cruzipain [16]. The enzyme had an apparent molecular mass of 41 kDa, as estimated by gel chromatography on a calibrated Superdex 75 HR column. Moreover, only one band, corresponding to 41 kDa, was visible in SDS-PAGE under non-reducing conditions (Fig. 1). The apparent molecular mass is compatible with the amino acid sequence of cruzipain [16] and the known glycosylation [3]. Under these experimental conditions anomalous electrophoretic migration giving different apparent molecular masses of the enzyme [31] was not observed.

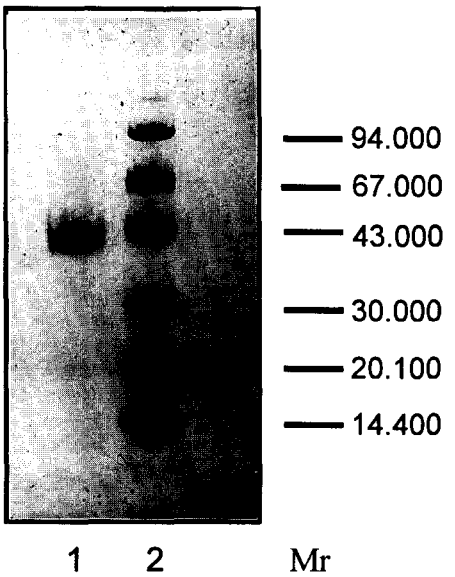

Fig. 1. SDS-PAGE of cruzipain under non-reducing conditions. Lane 1, cruzipain; Lane 2, protein standards.

Cruzipain appeared in multiple forms with pI values between 3.7 and 5.1 (Fig. 2), probably due to heterogeneity in both amino acid sequence and glycosylation in the C-terminal domain (J. Martínez, J. Henriksson, U. Petterson and J.J. Cazzulo, unpublished results).

The enzyme showed optimal activity at pH 6.5 against the fluorogenic substrate, Z-Phe-Arg-MCA, and was stable between pH 4.5 and 9.5. Cruzipain thus is more stable than lyso-

Table 1
Dissociation equilibrium constants (K_d), association rate constants (k_{ass}) and dissociation rate constants (k_{diss}) for the interaction between cruzipain and cysteine proteinase inhibitors

Inhibitor	$10^{12} \times K_d$ (M)	$10^{-6} \times k_{ass}$ (M^{-1} s^{-1})	$10^4 \times k_{diss}$ (s^{-1})
r stefin A	21.0 ± 0.2 (11)	1.7 ± 0.,2 (11)	0.36 ± 0.3*
stefin A	72.0 ± 1.2*	3.4 ± 0.2 (9)	2.5 ± 0.3 (9)
r stefin B	60.0 ± 1.0*	30.0 ± 0.2 (7)	18.0 ± 0.2 (7)
r human cystatin C	14.0 ± 0.4*	79.0 ± 0.5 (7)	10.0 ± 0.2 (7)
chicken cystatin	1.4 ± 0.2* 2.8 ± 0.2 (9)	73.0 ± 0.3 (8)	1.0 ± 0.1 (7)
L-kininogen	41.0 ± 0.8*	18.0 ± 0.1 (8)	7.4 ± 0.8 (8)

The experimental conditions are described in section 2. *Calculated from k_{ass} and K_d. *Calculated from k_{ass} and k_{diss}. Measured values are given with their standard errors and the number of measurements are given in parentheses.

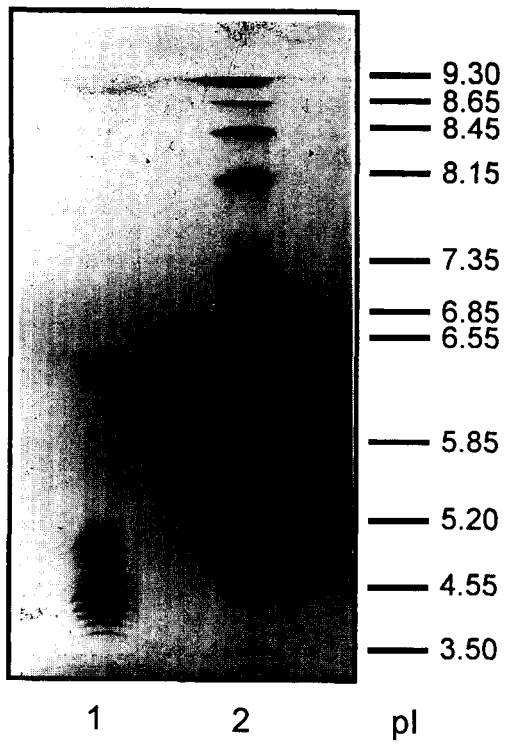

Fig. 2. Isoelectric focusing of pure cruzipain. Lane 1, cruzipain; Lane 2, protein standards.

somal cathepsins, which are inactivated under slightly alkaline conditions [28,32].

3.2. Kinetic measurements

Kinetic and equilibrium constants were determined for the interaction of the cysteine proteinase inhibitors, stefins A and B, cystatin C, chicken cystatin and kininogen, with cruzipain (Table 1). Association rate constants were measured under pseudo-first-order conditions by a continuous fluorimetric assay. Typical biphasic progress curves were recorded in all experiments. The dependence of the observed pseudo-first-order rate constant (k_{obs}) on inhibitor concentration was linear for all inhibitors, in agreement with a simple, competitive inhibition mechanism [29,30]. The association rate constants (k_{ass}) were calculated from the slope of the plot of k_{obs} vs. inhibitor concentration and were corrected for substrate competition. K_m value of 0.27 μM had been obtained. Dissociation rate constants (k_{diss}) were obtained either from the kinetic experiments, as described previously [24], or were measured by a displacement technique in the case of chicken cystatin [29]. Dissociation equilibrium constants, K_d, were then calculated from the association and dissociation rate constants as $K_d = k_{diss}/k_{ass}$. Alternatively, dissociation equilibrium constants for the interaction of cruzipain with stefin A and chicken cystatin were measured as inhibition constants, corrected for substrate competition as above [21]. The approximate equivalence in binding of cruzipain to natural and recombinant stefin A is indicated by the comparable dissociation constants obtained. The data further show that all inhibitors studied are highly active in inhibiting cruzipain. They bind rapidly and tightly to the enzyme with K_d values between ~2 and ~70 pM, cystatin C and chicken cystatin having somewhat higher affinities than the others. There are also some differences in the rates of association of the inhibitors with the enzyme, cystatin C and chicken cystatin reacting faster than stefin A.

In conclusion, the results of this study provide clear evidence that the cysteine proteinase from *T. cruzi* can be efficiently inhibited by host cystatins. A similar enzyme from *Trypanosoma congolense*, trypanopain, has also been shown to be inhibited by cystatins [33]. Parasite proteinases are attractive targets for drug design due to their key role in the parasite life cycles, as proved by the effects of inhibitors on the parasite's growth and differentiation, and in the pathogenesis of the diseases in host organisms, including humans. This has been shown for parasitic protozoa, ranging from *Entamoeba histolytica* [34], *Plasmodium falciparum* [35], *T. congolence* [33] to *T. cruzi* [9]. Therefore, the data presented here may be of significance for the design of new compounds as possible therapeutic agents for the treatment of Chagas' disease.

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