A fragment of the major histocompatibility complex class II – associated p41 invariant chain inhibits cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*

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Abstract A peptide fragment derived from the p41 form of the invariant chain (Ii) associated with the major histocompatibility complex (MHC) class II molecule has been shown to inhibit the mammalian lysosomal cysteine proteinase, cathepsin L, and to be a novel cysteine proteinase inhibitor, distinct from cystatins. Here we report that this same fragment also binds to and inhibits cruzipain, the cathepsin L-like enzyme from the protozoan parasite *Trypanosoma cruzi*. The binding of the Ii fragment to cruzipain is fast ($k_{ass} = 2.4 \times 10^7$ M^{-1} s^{-1}) and tight ($K_i = 5.8 \times 10^{-11}$ M). The inhibition is competitive. These results suggest the possibility of using the invariant chain as a model for the specific inhibition of cruzipain in vivo, i.e. as a potential drug to combat Chagas' disease.

Key words: Cruzipain; *Trypanosoma cruzi*; Invariant chain; p41; Cysteine proteinase inhibitor; Inhibition

1. Introduction

Cruzipain is the major cysteine proteinase of *Trypanosoma cruzi*, the parasitic flagellate which is the causative agent of American trypanosomiasis, Chagas' disease [1]. It is a lysosomal enzyme [2], also found at the cell surface of the parasite [3]. The mature enzyme consists of a catalytic domain homologous to cysteine proteinases from the papain family [4,5], and an unusual C-terminal extension, also present in related enzymes of other trypanosomatids [6]. The enzyme has been expressed in *E. coli*, and by self-processing of the precursor, active enzyme lacking the C-terminal extension (also called cruzain) was obtained [5] and crystallised [7]. The three-dimensional structure of the enzyme complexed with the irreversible inhibitor Z-Phe-Ala-fluoromethylketone has been solved, revealing that cruzipain is isostructural with papain [8] and suggesting a common catalytic mechanism [9].

Cruzipain is inhibited by a range of organomercurial, epoxysuccinyl and peptide aldehyde compounds [10,11], as well as by protein inhibitors belonging to the cystatin superfamily [12,13]. The latter bind tightly to the enzyme with K_i values between 1 and 72 pM [12].

The invariant chain (Ii) is an integral membrane protein which is associated with the major histocompatibility complex (MHC) class II heterodimer during early stages of its intracellular transport. It contributes critically to a proper presentation of antigens to $CD4^+$ T lymphocytes [14]. Recently, a novel property of Ii has been established. A 65 amino acid fragment from Ii exhibits strong inhibitory activity against cathepsin L ($K_i = 1.7$ pM), and also inhibits moderately cathepsin H ($K_i = 5.3$ nM) and papain (K_i=1.4 nM) [15]. It corresponds to the alternatively spliced fragment, which is present only in the minor, p41, but not in the major, p31, form of Ii. The inhibitory fragment shows no sequence homology to known cysteine proteinase inhibitors from the cystatin superfamily, and therefore represents a new class of inhibitors.

In this work we show that cruzipain is also inhibited by the alternatively spliced Ii fragment. The kinetics of this inhibition were studied in order to characterise the interaction between the parasitic proteinase and this new type of cysteine proteinase inhibitor.

2. Materials and methods

2.1. Materials

Cruzipain was purified as reported [12]. The Ii fragment, isolated as a complex with cathepsin L, was separated using reversed-phase HPLC [16], freeze-dried on a Speed-vac concentrator (model RC 100, Savant Instruments, Farmingdale, USA), and redissolved in distilled water.

Protein concentrations were determined according to the method of Lowry et al. [17].

The concentration of active cruzipain was determined using the cysteine proteinase inhibitor Ep-475, and the Ii fragment was titrated with active-site-titrated cathepsin L as previously described [15].

Ep-475 was purchased from Peptide Research Institute (Osaka, Japan), and Z-Phe-Arg-MCA from Serva (Heidelberg, Germany). Stock solution of the substrate was 1 mM Z-Phe-Arg-MCA in dimethylsulfoxide.

2.2. Determination of rate and equilibrium constants for the inhibition of cruzipain by the Ii fragment

The reaction mixture contained Ii fragment and substrate (Z-Phe-Arg-MCA) in 0.1 M phosphate buffer, pH 6.5, containing 1 mM EDTA. The reaction was started by the addition of 50 µl of cruzipain (preactivated with 8 mM DTE) to 1.950 ml of the reaction mixture. Final concentrations of the substrate and cruzipain were 5 µM and 200 pM, respectively, and the Ii fragment concentration varied from 2 to 5 nM. All the experiments were carried out at 24°C under pseudo-first-order conditions, with at least a 10-fold molar excess of the Ii fragment over cruzipain. Less than 5% of substrate was hydrolysed during each experiment. The release of product was monitored continuously at excitation and emission wavelengths of 370 and 460 nm, respectively, using a Perkin Elmer LS-50B spectrofluorimeter.

2.3. Determination of type of inhibition

The measurements were made under the same experimental condi-

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Abbreviations: C-, carboxyl-; DTE, dithioerythritol; E-64, L-3-carboxy-*trans*-2,3-epoxypropylleucylamido(4-guanidino)butane; Ep-475, L-3-carboxy-*trans*-2,3-epoxypropylleucylamido(3-guanidino)butane; Ii, invariant chain; -MCA, -4-methyl-7-coumarylamide; MHC, major histocompatibility complex; Z-, benzyloxycarbonyl-

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tions as described in Section 2.2 with a constant final concentration of cruzipain (80 pM) and of the Ii fragment (2 nM). The substrate concentration was varied from 0.25 to 1.25 μM.

3. Results

Under the experimental conditions used, all progress curves for the interaction between cruzipain and the Ii fragment showed an exponentially decreasing release of product with time (Fig. 1), following typical biphasic, slow-binding kinetics [18]. They were fitted by non-linear least-squares analysis to the following integrated rate equation [18]:

$$[P] = v_s t + (v_z - v_s)(1 - e^{-k_{obs}t})/k_{obs} \quad (1)$$

where [P] is the product concentration, and v_z and v_s are the initial and steady-state velocities, respectively. k_{obs} is the apparent pseudo-first-order rate constant describing the pre-steady-state of enzyme-inhibitor interaction, and is given by Eq. 2:

$$k_{obs} = k_{ass}[I_o]/(1 + [S_o]/K_m) + k_{diss} \quad (2)$$

where $[I_o]$ and $[S_o]$ represent inhibitor and substrate concentrations, K_m is the Michaelis-Menten constant, k_{ass} the second-order rate constant for enzyme-inhibitor complex formation, and k_{diss} the rate constant for its dissociation:

$$E + I \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} EI$$

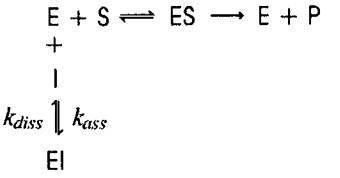
k_{obs} increased linearly with increasing concentration of the inhibitor, when substrate and enzyme concentrations were kept constant (Fig. 2). A k_{ass} value of $(2.4 \pm 0.14) \times 10^7$ $M^{-1}s^{-1}$ was calculated from the slope of the plot k_{obs} vs. $[I_o]$ [18] using a K_m value of 0.27 μM [12]. The dissociation rate constant k_{diss} was calculated for each inhibitor concentration using the following equation [18]:

$$k_{diss} = k_{obs} \times v_s/v_z \quad (3)$$

leading to an average value of $(1.4 \pm 0.27) \times 10^{-3}$ s^{-1}. The equilibrium inhibition constant K_i was calculated from $K_i = k_{diss}/k_{ass}$, giving $K_i = (5.8 \pm 1.4) \times 10^{-11}$ M.

In a control experiment with no inhibitor added, product formation was linear, verifying that the enzyme was stable during the experiment (Fig. 1).

The interaction of cruzipain and inhibitor was also investigated at different substrate concentrations with constant concentrations of enzyme and inhibitor. The k_{obs} value showed a linear dependence on $1/(1+[S_0]/K_m)$ (Fig. 3), which indicates a competitive inhibition mechanism:



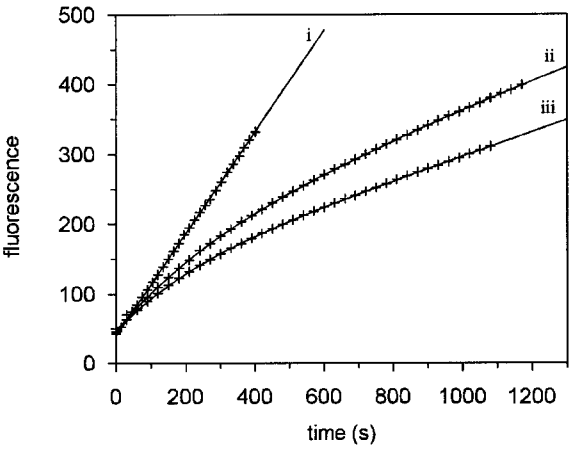


Fig. 1. Typical progress curves for the inhibition of cruzipain with the Ii fragment. $[E_o] = 200$ pM, $[S_o] = 5$ μM; $[I_o] = 0$ (i), $[I_o] = 2$ nM (ii), $[I_o] = 3$ nM (iii). Solid lines are theoretical curve fits (Eq. 1).

4. Discussion

Our experimental results show that the p41 invariant chain fragment is a potent inhibitor of cruzipain, the major cysteine proteinase from *T. cruzi*. The kinetic behaviour of these two molecules is consistent with a reversible, tight-binding inhibition mechanism [18]. The kinetic constants are of the same order of magnitude as those reported for the inhibition of cruzipain with cysteine proteinase inhibitors from the cystatin superfamily (Table 1), which also bind to cruzipain tightly with K_i values in the picomolar range [12].

On the other hand, the interaction between cruzipain and the Ii fragment is also similar to that of the same Ii fragment with cathepsin L [15]. The association rate constants for the interaction of cathepsin L and cruzipain with the Ii peptide are very similar, whereas the dissociation rate constant was considerably lower for cathepsin L (Table 1). The fact that cruzipain is inhibited to about the same extent as cathepsin L, whereas related enzymes are inhibited only weekly or not at

Table 1
Kinetic data for the interactions of cruzipain and of the Ii fragment

Enzyme	Inhibitor	k_{ass} ($\times 10^{-7}$) (M^{-1} s^{-1})	k_{diss} ($\times 10^5$) (s^{-1})	K_i (pM)
Cruzipain	Ii fragment	2.4 ±0.1	140 ±27	58 ±14
Cathepsin L[a]	Ii fragment	4.6 ±0.1	7.7 ± 1.0	1.7 ± 0.3
Cruzipain[b]	stefin A	0.34 ± 0.02	25 ±3	72.0 ± 1.2
Cruzipain[b]	stefin B	3.00 ± 0.02	180 ±2	60.0 ± 1.0
Cruzipain[b]	cystatin C	7.90 ± 0.05	100 ±2	14.0 ± 0.4
Cruzipain[b]	L-kininogen	1.80 ± 0.01	74 ±8	41.0 ± 0.8

[a]From [15].
[b]From [12].

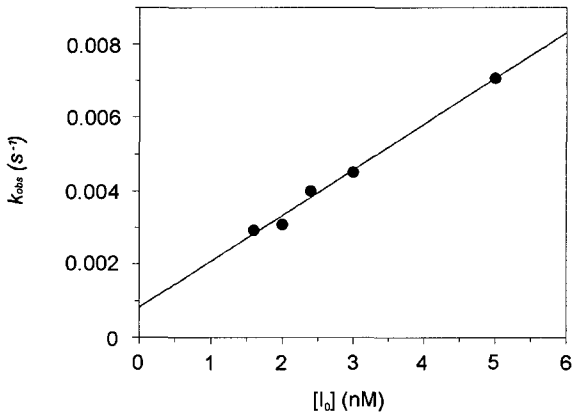


Fig. 2. Dependence of the apparent pseudo-first-order rate constant k_{obs} on inhibitor concentration for the interaction between cruzipain and the Ii fragment. $[E_o] = 200$ pM, $[S_o] = 5$ μM. The solid line was calculated by linear regression analysis.

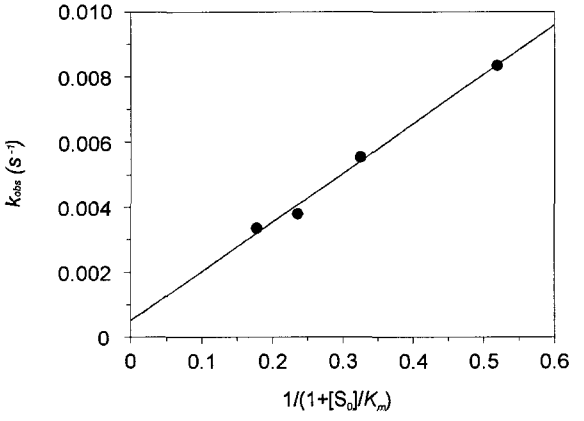


Fig. 3. Dependence of the apparent pseudo-first-order rate constant k_{obs} on substrate concentration for the interaction between cruzipain and the Ii fragment. $[E_o] = 80$ pM, $[I_o] = 2$ nM. The solid line was calculated by linear regression analysis.

all [15], is in agreement with previous observations [11,13] that the active site geometry of cruzipain resembles mostly cathepsin L.

The inhibitory Ii fragment shows no sequence homology with cystatins, but is homologous to thyroglobulin type-1 domain [19]. Recently, a protein from eggs of chum salmon has been isolated [20], which shares a high degree of homology with the thyroglobulin type-1 motif, and also inhibits cysteine proteinases. A novel class of cysteine proteinase inhibitors, distinct from cystatins, which had long been known as the only protein inhibitors of papain-like cysteine proteinases [21], is therefore emerging. The data presented here, together with the two other reports on these inhibitors [15,20] provide the starting point for building a model of their interaction with the target enzymes. The Ii fragment inhibits cruzipain in a competitive manner, which means that, like cystatins, it binds to the active site of cruzipain (Fig. 3).

At present, it is not possible to determine whether cruzipain inhibition by the Ii fragment could occur in vivo. One of the steps in the *T. cruzi* life cycle is penetration of the infective trypomastigotes into human macrophages. It could be that cruzipain and the p41 form of Ii (or a peptide derived from it) meet within the endosomal pathway of macrophages, which are known to be Ii-positive cells. In this case, the K_i value is sufficiently low to ensure complex formation at the expected physiological concentrations of enzyme and peptide.

Since cruzipain is a major cysteine proteinase from *T. cruzi*, substances which inhibit it might be useful as models in drug design for blocking the *T. cruzi* life cycle and thus providing a cure for Chagas' disease. The Ii fragment seems appropriate for this task because it shows higher specificity in its action compared to inhibitors from cystatin superfamily.

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