Biochem. J. (1996) **318**, 395–399 (Printed in Great Britain)

Biotin-labelled peptidyl diazomethane inhibitors derived from the substrate-like sequence of cystatin: targeting of the active site of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*

Gilles LALMANACH†, Roger MAYER*, Carole SERVEAU*, Julio SCHARFSTEIN‡ and Francis GAUTHIER*§

*Enzymologie et Chimie des Protéines, CNRS URA 1334, Faculté de Médecine, Université François Rabelais, 2bis Bd Tonnellé, 37032 Tours cedex, France,
†Glycobiologie, Centre de Biophysique Moléculaire, CNRS UPR 4301, 45071 Orléans cedex 2, France, and
‡Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21944 Rio de Janeiro, Brazil

Biotin-labelled peptidyl diazomethane inhibitors of cysteine proteinases, based on the N-terminal substrate-like segment of human cystatin C, a natural inhibitor of cysteine proteinases, were synthesized. These synthetic derivatives were tested as irreversible inhibitors of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*, to compare the kinetics of the inhibition of the parasite proteinase with that of the mammalian cathepsins B and L. The accessibility of the active sites of these proteinases to these probes was also investigated. The inhibition of cruzipain by Biot-LVG-CHN_2 (where Biot represents biotinyl and L,V and G are single-letter amino acid residue abbreviations) and Biot-Ahx-LVG-CHN_2 (where Ahx represents 6-aminohexanoic acid) was similar to that of unlabelled inhibitor. Biotin labelling of the inhibitor slowed the inhibition of both cathepsin B and cathepsin L. Adding a spacer arm (Ahx) between the biotin and the peptide moiety of the derivative increased the inhibition of cathepsin B but not that of cathepsin L. The discrimination provided by this spacer is probably due to differences in the topologies of the binding sites of proteinases, a feature that can be exploited to improve targeting of individual cysteine proteinases. Analysis of the blotted proteinases revealed marked differences in the accessibility of extravidin–peroxidase conjugate to the proteinase-bound biotinylated inhibitor. Cruzipain molecules exposed to Biot-LVG-CHN_2 or Biot-Ahx-LVG-CHN_2 were readily identified, but the reaction was much stronger when the enzyme was treated with the spacer-containing inhibitor. In contrast with the parasite enzyme, rat cathepsin B and cathepsin L treated with either Biot-LVG-CHN_2 or Biot-Ahx-LVG-CHN_2 produced no detectable bands. Papain, the archetype of this family of proteinases, was poorly labelled with Biot-LVG-CHN_2, but strong staining was obtained with Biot-Ahx-LVG-CHN_2. These findings suggest that optimized biotinylated diazomethanes might considerably improve their selectivity for the *T. cruzi* target enzyme.

INTRODUCTION

Evidence has accumulated over the past few years showing that cysteine proteinases from helminths and protozoans are essential in parasite development [1,2]. The papain-like proteinases expressed by the intracellular parasite *Trypanosoma cruzi* (designated as cruzipain, cruzain or GP57/51) is recognized as a potential target for chemotherapy against Chagas' disease [3,4], and a great deal is known of their biochemical and structural properties [5–8]. The cruzipains are encoded by multicopy polymorphic genes and are functionally and structurally similar to mammalian cathepsin L [9,10]. As a distinctive feature, however, they contain a proteolytically sensitive proline-rich type of hinge structure followed by a contiguous polythreonine sequence [11,12], which separates the catalytic domain and a long (130 residues), highly antigenic C-terminal extension [11,13]. Like mammalian cathepsins, the cruzipain(s) are synthesized as single-chain pre-proproteins [6]. Their expression is developmentally regulated, reaching high levels during the replicating stages of parasite development [6,9,14]. The three-dimensional structure of the catalytic domain of recombinant cruzain co-crystallized with its inhibitor Z-Phe-Ala-FMK (where Z represents benzyloxycarbonyl) has recently been resolved, providing a valuable guide for detailed investigations of the conformation and specificity of the active site [8]. Further information on target selectivity is, however, needed to exploit the potent trypanocidal effects of synthetic peptidyl inhibitors on infected cultures [3,4] and in animal models of Chagas' disease [8]. The first-generation peptidyl diazomethane and peptidyl fluoromethane inhibitors have limited selectivity for the parasite enzyme because they were originally developed for papain and closely related mammalian proteinases (reviewed in [15]). Prompted by the observation that cruzipain is strongly inhibited by several members of the cystatin superfamily [16,17], we recently designed peptide substrates with intramolecularly quenched fluorescence and peptidyl diazomethane inhibitors from the N-terminal substrate-like segment of cystatin C, and from the conserved QxVxG sequence in cystatins [18–20]. Although some of these substrates showed increased specificity for cruzipain, the peptidyl diazomethanes Z-RLVG-CHN_2 and GlcA-QVVA-CHN_2 still inhibited papain and rat cathepsins B and L more efficiently [17,18]. We have attempted to overcome these limitations by exploring the active site accessibility of cystatin-based diazomethane inhibitors by labelling the N-terminus of the peptidyl portion with biotin, or using a 6-aminohexanoate spacer to increase the distance between the biotin and the peptidyl core of the inhibitor. The underlying premise was that cysteine proteinases might have different active-

Abbreviations used: AMC, 7-amino-4-methylcoumarin; Ahx, 6-aminohexanoic acid; Biot, biotinyl; DTT, dithiothreitol; Z, benzyloxycarbonyl.

§ To whom correspondence should be addressed.

site topologies that could favour or restrict the access of the biotinylated inhibitor to the cleft. The kinetics of the interaction of the biotinylated derivatives with mammalian cathepsins B and L and cruzipain (isolated from the Dm28c strain of *T. cruzi*) were compared. The results indicate that the active site of cruzipain is more accessible to these probes than are its mammalian counterparts. In addition to their implications for the design of inhibitors, these probes might help to locate catalytically active cruzipain in viable organisms.

EXPERIMENTAL

Materials

Prestained molecular mass markers were from Bio-Rad (Ivry-sur-Seine, France). Dithiothreitol (DTT) and BSA were obtained from Sigma (St. Quentin-le-Fallavier, France). Z-Phe-Arg-AMC (where AMC represents 7-amino-4-methylcoumarin) and Z-Arg-Arg-AMC were purchased from Bâle Biochimie (Voisins-le-Bretonneux, France). Extravidin–peroxidase conjugate and 4-chloro-1-naphthol were from Sigma. Unless otherwise stated, all amino acids were of the L-configuration and were from Bachem (Budendorf, Switzerland). Biotin-*N*-hydroxysuccinimide ester and biotinamido-6-hexanoate-*N*-hydroxysucinimide ester were purchased from Pierce (Interchim, Montluçon, France). TLC was performed on Merck silica gel plates. All other reagents were of analytical grade.

Synthesis of peptidyl diazomethanes

Z-RLVG-CHN_2 was a gift from Dr. Magnus Abrahamson (University Hospital, Lund, Sweden) [21]. Biot-LVG-CHN_2 (where Biot represents biotinyl) and Biot-Ahx-LVG-CHN_2 (where Ahx represents 6-aminohexanoic acid) were synthesized from t-butoxycarbonyl-LVG-OMe, prepared by standard peptide chemistry. The N-biotinyl active ester moiety was appended to HCl,H-LVG-OMe in dimethylformamide in the presence of di-isopropylethylamine. The diazomethane peptides were obtained from the corresponding biotinylated peptide methyl ester, after saponification of the methyl ester with 1 M NaOH in DMSO by the method of Green and Shaw [22]. Peptides were purified by reverse-phase HPLC, and characterized by proton NMR with a Bruker AM 300 spectrometer.

Enzymes

Papain was purchased from Boehringer (Mannheim, Germany). Cathepsins B and L were purified from rat liver [23]. Cruzipain (Dm28c strain) was purified from epimastigotes of *T. cruzi* [24].

Cathepsin B was assayed on Z-Arg-Arg-AMC, cathepsin L, cruzipain and papain on Z-Phe-Arg-AMC, and their enzymic activities were recorded on a Kontron SFM 25 spectrofluorimeter with excitation at 350 nm and emission at 460 nm. These assays were conducted in activating buffer (0.1 M phosphate buffer, pH 6.0, containing 1 mM EDTA, 2 mM DTT and 0.1 % Brij 35) for cathepsins B and L, in 0.1 M phosphate buffer, pH 6.8, containing 1 mM EDTA, 2 mM DTT and 0.1 % Brij 35 for papain, and in 0.1 M phosphate buffer, pH 6.0, containing 10 mM DTT for cruzipain. Cysteine proteinases were titrated with *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane [25].

Kinetics

Studies were performed by continuous assay by the method of Tian and Tsou [26]. Briefly, the inactivation of proteinases by inhibitors was monitored in the presence of the substrate, allowing competition for the enzyme binding site. In this system, product formation in the presence of an irreversible inhibitor approaches an asymptote, as described by:

$$\log([P]_\infty - [P]) = \log[P]_\infty - 0.43k_{obs}t \quad (1)$$

where $[P]_\infty$ is the concentration of product formed at a time approaching infinity, [P] is the concentration of product at time t, k_{obs} is an apparent pseudo-first-order rate constant that relates to k_{ass}, the second-order rate constant for association of the inhibitor with the enzyme in the presence of the substrate as described by:

$$k_{obs} = k_{ass}[I]/(1+[S]/K_m) \quad (2)$$

where [I] and [S] are the inhibitor and substrate concentrations respectively, and K_m is the Michaelis–Menten constant. The second-order rate constant for inhibition, k_{ass}, is deduced from the slope of a plot of $\log([P]_\infty - [P])$ against t in accordance with eqn. (1).

Reactions were performed under experimental conditions where less than 10 % of the substrate was hydrolysed before the proteinase was inactivated. Mixtures of 25 μM Z-Phe-Arg-AMC and peptidyl diazomethane (0–50 μM) were preincubated for 2 min at 30 °C in a final volume of 400 μl. The enzyme reaction was started by adding 0.2 nM cruzipain, and the rate of substrate hydrolysis was monitored until inhibition was complete. The native enzyme was also checked to ensure that it retained full proteolytic activity under the experimental conditions. The inactivation of cathepsins B and L was recorded under similar conditions.

Detection of affinity-labelling probes

Enzymes (0.1 μM) were incubated in their respective activating buffers with a large excess of peptidyl diazomethane (molar ratio 1:200) for 1 h at 30 °C. Samples (10 μl of a 50 nM enzyme solution) were subjected to SDS/PAGE [15 % (w/v) gels] under reducing conditions [27]. Proteins were blotted by electro-transfer, and the nitrocellulose sheet was saturated by incubation for 1 h at 37 °C with PBS containing 1 % (w/v) BSA. The sheet was washed three times with PBS containing 0.1 % Tween-20, and incubated with extravidin–peroxidase for 2 h at room temperature under gentle agitation, diluted 1:500 in PBS containing 3 % (w/v) BSA and 0.05 % Tween-20. It was again washed three times and the peroxidase activity was revealed with 4-chloro-1-naphthol (Sigma) or with the enhanced chemiluminescence kit from Amersham. Parallel experiments were performed by dot blotting with and without prior reduction of the enzyme–inhibitor complex.

RESULTS AND DISCUSSION

Targeting the active site of parasite proteinases during infection of the host without impairing the functioning of host proteins is a new and promising approach for developing chemotherapeutic agents against parasitic disesases [28]. However, no specific cruzipain substrate or inhibitor has yet been described that discriminates between this proteinase and related host cysteine proteinases during infection. We have previously shown the important contribution of residues in prime position with respect to the substrate cleavage site to cruzipain specificity [17], a feature that runs counter to the development of proteinase-specific peptidyl diazomethane or peptidyl fluoromethane inhibitors. An alternative method of specific targeting of cruzipain is to assume that the access of peptide inhibitors to the proteinase

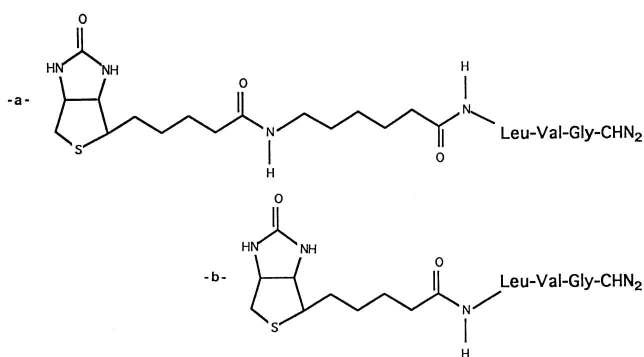

Figure 1 **Structure of the biotinylated probes: (a) Biot-Ahx-LVG-CHN$_2$; (b) Biot-LVG-CHN$_2$**

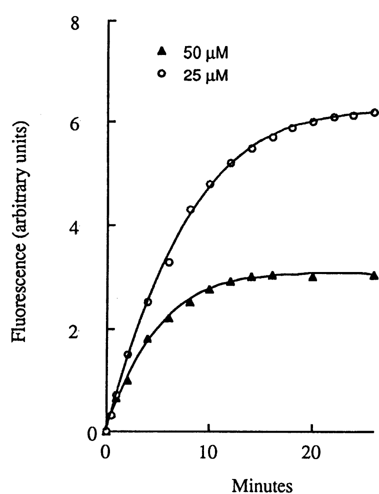

Figure 2 Progress curves for the generation of 7-amino-4-methylcoumarin from the cruzipain-catalysed hydrolysis of Z-Phe-Arg-AMC in the presence of Biot-LVG-CHN$_2$

The inactivation of cruzipain was measured by continuous monitoring of the increase of fluorescence, as described in the Experimental section, in the presence of a fixed concentration of substrate (25 μM) and various amounts of Biot-LVG-CHN$_2$, as described in [17].

Table 1 Second-order inhibition constants (k_{ass}) of cruzipain and rat lysosomal cathepsins by peptidyl diazomethanes

Second-order constants were determined as described in the Experimental section.

	k_{ass} ($M^{-1} \cdot s^{-1}$)		
	Z-RLVG-CHN$_2$	Bio-LVG-CHN$_2$	Bio-Ahx-LVG-CHN$_2$
Cruzipain	5900*	1500	1200
Cathepsin L	241000†	7500	16000
Cathepsin B	31000†	100	7000

* Results taken from [17].
† Results taken from [18].

active site could be modulated by a bulky hydrophobic derivative such as biotin, because of the variability of the amino acid sequence within the active site of cysteine proteinases, and because of the somewhat different conformations of the active sites of those cysteine proteinases whose three-dimensional structures are known (reviewed in [29]). This would provide further discrimination between related cysteine proteinases of the papain family, and help to locate the parasite proteinase *in situ*.

Inhibition of cysteine proteinase by biotinylated inhibitors

Cystatin-derived peptidyl diazomethane inhibitors were N-terminally labelled with biotin, directly or via a spacer arm, to investigate the capacity of the probe to access the active site (see Figure 1). Biotin-labelled inhibitors were first assayed kinetically on rat cathepsins B and L and on cruzipain. The rate constants for inactivation were compared with those obtained with unlabelled inhibitors. Figure 2 shows the time course for the formation of 7-amino-4-methylcoumarin, released from Z-Phe-Arg-AMC by cruzipain from the Dm28c clone, in the presence of Biot-LVG-CHN$_2$. The observation that the curves obtained at two different inhibitor concentrations have the same initial slopes suggests strongly that no significant non-covalent enzyme–inhibitor binding occurs during the course of competitive, irreversible inhibition, and therefore that the mechanism obeys the simple model:

$$E + I \xrightarrow{k_{ass}} EI \tag{3}$$

This was further confirmed by plotting k_{obs} against [I], which gave a straight line (not shown). Similar results were obtained with cathepsins B and L, indicating that biotinylated inhibitors still have access to the active site of proteinases. However, the rate at which inhibition occurs changes markedly with the proteinase used (Table 1). Biot-LVG-CHN$_2$ inactivated cathepsin L at one-thirtieth of the rate of the unlabelled inhibitor. The ratio was about 1:300 for cathepsin B. However, the rate of cruzipain inhibiton was only slightly decreased by the biotinylated inhibitor. Parallel experiments using papain showed that biotinylation significantly increased (4-fold) the rate of inhibition (results not shown). Biotinylation might therefore selectively affect the access of the peptidyl diazomethane to the active site of papain-related proteinases. This finding could be used as the platform on which to develop more selective probes for the parasite proteinase, using the relatively low sensitivity of cruzipain to biotinylation of the inhibitor compared with its mammalian homologues. How biotin might impair access to the active site of cathepsin B and L was further investigated by introducing an alkylated spacer between the biotin group and the peptide chain. The rate of inhibition of cruzipain and cathepsin L was not significantly changed by using Biot-Ahx-LVG-CHN$_2$. However, the presence of the spacer arm greatly increased the rate at which cathepsin B was inhibited (Table 1). Whether this is due to interactions of biotin with critical residues in the substrate-binding site or whether it is due to an unfavourable environment within the active site of cathepsin B cannot be answered yet. We therefore studied the accessibility of biotin to extravidin after proteinase–inhibitor complexes were formed. This affinity labelling could help to discriminate further between cysteine proteinases and be used as a first step to identify mature forms of cysteine proteinases in living cells or tissues.

Affinity labelling of cysteine proteinases

Rat cathepsins B and L, and Dm28c cruzipain bound to biotinylated inhibitors, were revealed by extravidin–peroxidase conjugates after Western blotting. Only the cruzipain-bound

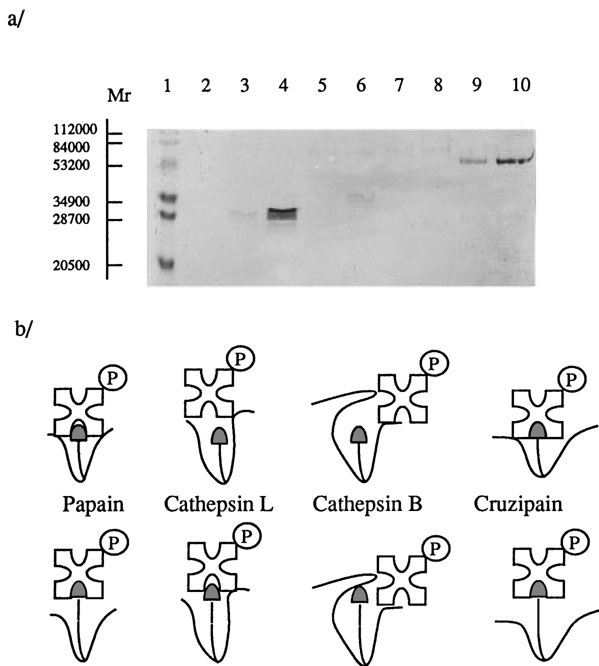

Figure 3 Interaction between cystein proteinases and biotinylated diazomethane inhibitors

(**a**) Affinity-labelling of cysteine proteinases by the biotinylated cystatin-derived probes. Enzymes were incubated with Biot-LVG-CHN_2 or Biot-Ahx-LVG-CHN_2, and electroblotted after SDS/PAGE (see the Experimental section).The nitrocellulose sheet was incubated with extravidin–peroxidase conjugate (1 : 500) and revealed with 4-chloro-1-naphthol. Each sample corresponds to 10 μl of a 50 nM enzyme solution. Samples are: lane 1, pre-stained molecular mass markers (Biorad); lane 2, cruzipain/Z-RLVG-CHN_2; lane 3, papain/Biot-LVG-CHN_2; lane 4, papain/Biot-Ahx-LVG-CHN_2; lane 5, cathepsin L/Biot-LVG-CHN_2; lane 6, cathepsin L/Biot-Ahx-LVG-CHN_2; lane 7, cathepsin B/Biot-LVG-CHN_2; lane 8, cathepsin B/Biot-Ahx-LVG-CHN_2; lane 9, cruzipain/Biot-LVG-CHN_2; lane 10, cruzipain/Biot-Ahx-LVG-CHN_2. (**b**) Schematic drawing of the interaction between cysteine proteinases and biotinylated inhibitors without (top) and with (bottom) the spacer arm, illustrating the different accessibilities of the active site of each proteinase. Peptidyl diazomethanes are represented by the shaded stemmed structure, and peroxidase-labelled extravidin is represented as a four-armed structure.

biotin remains accessible to the conjugate, regardless of the probe used. The staining, however, was more pronounced with Biot-Ahx-LVG-CHN_2 (Figure 3a). Preincubation of cruzipain with Z-RLVG-CHN_2 completely blocked binding of the biotin label by this proteinase, confirming the specific targeting of the active-site-reactive thiol by the inhibitor. Cathepsin B was not labelled by either Biot-LVG-CHN_2 or Biot-Ahx-LVG-CHN_2 under the conditions used, and cathepsin L was only faintly labelled by incubation with the longer diazomethane (Figure 3a). Therefore selective identification of the parasite proteinases could be obtained via affinity labelling with cystatin-derived inhibitors. These results might indicate significant differences in the topology of the active site of cysteine proteinases. Like cruzipain, papain was labelled by both inhibitors, but was much better stained by Biot-Ahx-LVG-CHN_2 than by Biot-LVG-CHN_2 (Figure 3a). Similar results were obtained by detection of chemiluminescence, showing that the non-detection of mammalian cathepsins was not due to the low sensitivity of the staining method. Analysis by dot-blotting of the samples, with or without prior reduction, gave the same results (not shown). The structural basis for the different reactions of cruzipain and its mammalian counterparts remains unclear. The results of Western blotting are summarized in Figure 3(b). The non-recognition of the biotinylated inhibitor complexed with cathepsin B could be due to the occluding loop in the vicinity of the active site [30], preventing extravidin from binding to the biotin in the enzyme–inhibitor complex. However, peptidyl diazomethane labelled with biotin on its P2 site (Biot-Phe-Ala-CHN_2) have been shown to remain accessible to streptavidin–alkaline phosphatase when the inhibitor is bound to cathepsin B [31]. The three-dimensional structure of cathepsin L is not yet available but this proteinase has no occluding loop, similar to that of cathepsin B, that would explain the poor accessibility of the biotinylated inhibitor bound to the proteinase.

A recent crystallographic study has shown that the unprimed specificity of papain is determined by S1 to S3 subsites, and that the P4 residue in substrates or inhibitors protudes from the papain-binding site, and interfaces with solvent molecules without any specific interactions [32]. This agrees with previous molecular modelling studies describing the interaction between papain and a cystatin-derived tetrapeptide [20]. The observation that a biotin in P4 does not prevent the irreversible inhibitor from binding to the enzyme active site also agrees with this finding. Because of its hydrophobicity, the protruding biotin in proteinase–diazomethane complexes could interact with hydrophobic surface residues. In the avidin–biotin complex the bulky heterocycle of the biotinyl moiety sandwiches tightly with aromatic rings in avidin [33]. There could be favourable stacking interactions between the terminal part of the probe and aromatic residues in the vicinity of the active site. Hence the accessibility of the enzyme–biotinylated inhibitor complex to the extravidin–peroxidase conjugate might depend on the fine structural features of each target proteinase.

The ability of biotinylated peptidyl diazomethane inhibitors, derived from the cystatin sequence, to specifically label cruzipain makes them versatile tools for targeting the parasite proteinase in mammalian cells infected by *T. cruzi*. Preliminary studies on living forms of *T. cruzi* indicate that these biotinylated inhibitors are targeted to intracellular vesicles, their localization coinciding with that of cruzipains (A. C. dos Santas Monteiro, M. A. Vannier-Santos, G. Lalmanach, A. P. C. A. Lima, F. Gauthier and J. Scharfstein, unpublished work).

We thank Dr. Magnus Abrahamson for his gift of Z-RLVG-CHN_2, and Emmanuelle Mothes for help with inhibitor synthesis. We thank Dr. Thierry Moreau for helpful discussion. C.S holds an MRES doctoral fellowship. This work was supported by a grant from Biotechnocentre and by PADCT-CNPq.

REFERENCES

1 North, M. J., Mottram, J. C. and Coombs, G. H. (1990) Parasitol. Today **6**, 270–275
2 MacKerrow, J. H., Sun, E., Rosenthal, P. J. and Bouvier, J. (1993) Annu. Rev. Microbiol. **47**, 821–853
3 Meirelles, M. N. L., Juliano, L., Carmona, E., Silva, S. G., Costa, E. M., Murta, A. C. M. and Scharfstein, J. (1992) Mol. Biochem. Parasitol. **52**, 175–184
4 Harth, G., Andrews, N., Mills, A. A., Engel, J. C., Smith, R. and MacKerrow, J. H. (1993) Mol. Biochem. Parasitol. **58**, 17–24
5 Cazzulo, J. J., Couso, R., Raimondi, A., Wernstedt, C. and Hellman, U. (1989) Mol. Biochem. Parasitol. **33**, 33–42
6 Eakin, A. E., Mills, A. A., Harth, G., MacKerrow, J. H. and Craik, C. S. (1992) J. Biol. Chem. **267**, 7411–7420
7 Murta, A. C. M., Persechini, P. M., Souto-Padron, T., De Souza, W., Guimaraes, J. A. and Scharfstein, J. (1990) Mol. Biochem. Parasitol. **43**, 27–38
8 McGrath, M. E., Eakin, A. E., Engel, J. C., MacKerrow, J. H., Craik, C. S. and Fletterick, R. J. (1995) J. Mol. Biol. **247**, 251–259
9 Campetella, O., Henriksson, J., Aslund, L., Frasch, A. C. C., Pettersson, U. and Cazzulo, J. J. (1992) Mol. Biochem. Parasitol. **50**, 225–234
10 Lima, A. P. C. A., Tessier, D. C., Thomas, D. Y., Scharfstein, J., Storer, A. C. and Vernet, T. (1994) Mol. Biochem. Parasitol. **67**, 333–338
11 Hellman, U., Wernstedt, C. and Cazzulo, J. J. (1991) Mol. Biochem. Parasitol. **44**, 15–22
12 Aslund, L., Henriksson, J., Campetella, O., Frasch, A. C. C., Pettersson, U. and Cazzulo, J. J. (1991) Mol. Biochem. Parasitol. **45**, 345–348

13 Scharfstein, J., Rodrigues, M. M., Alves, C. A., De Souza, W., Previato, J. O. and Mendonca-Previato, L. (1983) J. Immunol. **131**, 972–977
14 Bonaldo, M.C, D'Escoffier, L. N., Salles, J. M. and Goldenberg, S. (1991) Exp. Parasitol. **73**, 44–51
15 Shaw, E. (1990) Adv. Enzymol. **63**, 271–347
16 Stoka, V., Nycander, M., Lenarcic, B., Labriola, C., Cazzulo, J. J., Björk, I. and Turk, V. (1995) FEBS Lett. **370**, 101–104
17 Serveau, C., Lalmanach, G., Juliano, M. A., Scharfstein, J., Juliano, L. and Gauthier, F. (1996) Biochem. J. **313**, 951–956
18 Lalmanach, G., Serveau, C., Brillard-Bourdet, M., Chagas, J. R., Juliano, L., Mayer, R. and Gauthier, F. (1995) J. Protein Chem. **14**, 645–653
19 Gauthier, F., Moreau, T., Lalmanach, G., Brillard-Bourdet, M., Ferrer-Di Martino, M. and Juliano, L. (1993) Arch. Biochem. Biophys. **306**, 304–308
20 Serveau, C., Juliano, L., Bernard, P., Moreau, T., Mayer, R. and Gauthier, F. (1994) Biochimie **76**, 153–158
21 Hall, A., Abrahamson, M., Grubb, A., Trojnar, J., Kania, P., Kasprzykowska, R. and Kasprzykowski, F. (1992) J. Enzyme Inhib. **6**, 113–123
22 Green, G. D. J. and Shaw, E. (1981) J. Biol. Chem. **256**, 1923–1928
23 Moreau, T., Gutman, N., El Moujahed, A., Esnard, F. and Gauthier, F. (1988) Eur. J. Biochem. **173**, 185–190
24 Lima, A. P. C. A., Scharfstein, J., Storer, A. C. and Ménard, R. (1992) Mol. Biochem. Parasitol. **56**, 335–338
25 Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. and Hanada, K. (1982) Biochem. J. **201**, 189–198
26 Tian, W. X. and Tsou, C. L. (1982) Biochemistry **21**, 1028–1032
27 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
28 McKerrow, J. H., McGrath, M. E. and Engel, J. C. (1995) Parasitol. Today **11**, 279–282
29 Rawlings, N. D. and Barrett, A. J. (1994) Methods Enzymol. **244**, 461–486
30 Musil, D., Zucic, D., Engh, R. A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and Bode, W. (1991) EMBO J. **10**, 2321–2330
31 Walker, B., Cullen, B. M., Kay, G., Halliday, I. M., McGinty, A. and Nelson, J. (1992) Biochem. J. **283**, 449–453
32 Matsumoto, K., Murata, M., Sumiya, S., Kitamura, K. and Ishida, T. (1994) Biochim. Biophys. Acta **1208**, 268–276
33 Livnah, O., Bayer, E. A., Wilchek, M. and Sussman, J. L. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5076–5080

Received 19 February 1996/7 May 1996; accepted 29 May 1996