Substrate inhibition of cruzipain is not affected by the C-terminal domain

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Abstract Endogenous and recombinant cruzipain, the major cysteine proteinase from the protozoan parasite *Trypanosoma cruzi*, exhibit differences in the protein and circular dichroism spectra probably attributed to the absence of the C-terminal domain in the recombinant enzyme. Substrate hydrolysis of both molecules at 25°C and neutral pH obeyed Michaelis-Menten kinetics whereas significant substrate inhibition was observed above neutral pH. The results suggest that substrate inhibition of cruzipain is pH-dependent, and that the C-terminal domain does not play an essential role in this process.

Key words: Cruzipain; Cysteine proteinase; Substrate inhibition; C-terminal domain; Substrate specificity; *Trypanosoma cruzi*

1. Introduction

Cysteine proteinases belonging to the papain superfamily are present in all groups of eukaryotes, except fungi [1]. Most of the proteinase activity detected in trypanosomatids (*Trypanosoma cruzi*, *T. brucei*) and various *Leishmania* spp. has been characterized as type I cysteine proteinases which are cathepsin L-like enzymes with an unusual C-terminal extension [2,3]. A C-terminal domain [4] is also found in plants such as tomato [5] and rice [6]. The length of the C-terminal extension ranged from 100 to 130 amino acids [2,7–9], and the sequence between the catalytic domain and the C-terminal domain, containing clusters of Thr, Pro and Ser, is susceptible to proteolytic cleavage [2,4,5]. The function of the C-terminal domain is unknown. It has been proposed to be involved in the evasion of the immune response of the host [10] and enhancement of the catalytic activity [11]. Other hypotheses on its role in targeting of the enzyme to the lysosomes or protein folding have been excluded [3,8].

Cruzipain (cruzain, gp 57/51) is the major protease of the protozoan parasite *T. cruzi* [12]. The cruzipain gene encodes a protein which contains a prepro region, a catalytic domain and a C-terminal domain [7,8]. Recombinant cruzipain has been expressed in *Escherichia coli* [8]. After complete proteolytic processing only the catalytic domain of the enzyme was recovered [13]. Using small synthetic substrates, cruzipain showed temperature-dependent substrate inhibition which was suggested to be due to its C-terminal domain [14].

In order to define if the C-terminal domain plays a major role in substrate inhibition or other catalytic properties of the protease, studies were carried out on endogenous and recombinant cruzipain molecules, containing or lacking the C-terminal domain. Circular dichroism (CD) spectra of both proteins were scanned for comparison. The complete pH profile for the second order rate constant k_{cat}/K_m for the hydrolysis of Z-Phe-Arg-MCA and Z-Arg-Arg-MCA by cruzipain has been determined.

2. Materials and methods

Dithioerythritol and EDTA were purchased from Sigma (USA). HEPES, glycine and MCA were from Serva (Germany). Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were obtained from Bachem (Switzerland). E-64 was provided by the Peptide Research Institute (Japan). Dimethyl sulfoxide was from Merck (Germany). All other chemicals were of analytical grade. Stock solutions of substrates and inhibitor were prepared in dimethyl sulfoxide.

2.1. Enzyme purification

Cruzipain was isolated from *T. cruzi* epimastigotes, Tulahuén strain, Tul 2 stock [15]. A truncated variant of recombinant cruzipain lacking the C-terminal domain was expressed in *E. coli* as a fusion protein as described [13]. The fully processed recombinant enzyme was further purified on a Superdex 75 HR column (Pharmacia, Sweden) equilibrated with 100 mM phosphate buffer, 1 mM EDTA, 300 mM NaCl, pH 7.0. The enzyme activity was measured using Z-Phe-Arg-MCA substrate. Enzymatically active fractions were pooled, concentrated and stored at −20°C prior to use. The active concentration of the enzymes was determined by active site titration with E-64 [16].

2.2. Protein concentration

Protein concentration was determined by absorbance measurements at 280 nm in a Perkin Elmer UV/VIS Spectrometer Lambda 18 (USA). Molar absorption coefficient values of 58 285 M^{-1} cm^{-1} for endogenous cruzipain and 54 930 M^{-1} cm^{-1} for recombinant cruzipain were calculated from amino acid sequences [17].

2.3. Circular dichroism

CD spectra were recorded using an AVIV 62 DS spectropolarimeter at 25°C. A cell with 0.1 cm path length was used for far-UV (190–250 nm) measurements and a 1 cm path length cell for the near-UV region (250–340 nm). Data were collected every 0.5 nm for the far-UV and every 1.0 nm for the near-UV regions, with a time constant of 2 s and a spectral bandwidth of 0.5 nm. Each spectrum was the average of three repetitive scans corrected for cell and solvent contributions. The CD spectra of endogenous and recombinant cruzipain were scanned under native conditions at pH 7.0. The enzyme concentration was 7 μM. In the far-UV CD the results are reported in terms of mean residue ellipticity $[\theta]_{MRW}$ defined as $[\theta]_{MRW} = [\theta] \times MRW/(10 \times c \times l)$ where θ is the ellipticity in millidegrees, c is the concentration in mg/ml and l is the light path length in cm. MRW is the mean residue weight, 105 for endogenous cruzipain and 110 for recombinant cruzipain. In the near-UV CD the results are reported in molarity. The

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Abbreviations: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; E-64, 1-[L-*N*-(*trans*-epoxysuccinyl)leucyl]amino-4-guanidinobutane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MCA, 4-methyl-7-coumarylamide; Z, benzyloxycarbonyl

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secondary structure composition was estimated by the CONTIN algorithm [18].

2.4. Dependence of the rate of hydrolysis on substrate concentration

Enzyme samples were activated with dithioerythritol (2 mM final concentration) for 5 min at 25°C. 10 μl of the activated sample (52 nM final concentration) was added to 390 μl of Z-Phe-Arg-MCA (0.1–200 μM) dissolved in 100 mM phosphate buffer (Na_2HPO_4/KH_2PO_4), pH 7.0 or in 50 mM glycine buffer, pH 9.0, both containing 1 mM EDTA and 100 mM NaCl. The progress of the reaction was followed continuously at 25°C for 20 s using Perkin Elmer Luminescence Spectrometer LS 50 (USA) with excitation and emission wavelengths of 370 and 460 nm, respectively. Enzymes were shown to be stable during the measurements. In all experiments, dimethyl sulfoxide content was adjusted to 3% and substrate consumption was limited to less than 5%.

2.5. pH dependence of k_{cat}/K_m

pH dependence studies were carried out in buffers prepared by mixing the following solutions in the appropriate ratio: 100 mM citric acid and 200 mM $Na_2HPO_4{\cdot}2H_2O$ (pH 2.2–8.0), 50 mM $Na_2B_4O_7{\cdot}10H_2O$ and 100 mM HCl (pH 8.0–9.0), 50 mM $Na_2B_4O_7{\cdot}10H_2O$ and 100 mM NaOH (pH 9.0–10.5). All buffers contained 1 mM EDTA and 100 mM NaCl. 10 μl of activated cruzipain (0.1 μM) was added to 490 μl of Z-Phe-Arg-MCA (0.1–1 μM) or Z-Arg-Arg-MCA (0.5–3 μM) in buffer solutions from pH 2.7 to 10.2 at 25°C. Due to the low signal/noise ratio for the measurements performed in the presence of Z-Arg-Arg-MCA substrate below pH 4.5, 20 μl of activated cruzipain (4.8 μM) was added to 480 μl of substrate. Enzyme activity was measured as described above. The fluorimeter was calibrated with 0.25 μM MCA in the same buffer solutions.

3. Results and discussion

3.1. Protein spectra

The protein spectrum of endogenous cruzipain exhibits a profile with a maximum at 280 nm whereas an unusual 12 nm red shift of its maximum was observed on recombinant cruzipain (Fig. 1). The primary structure of cruzipain [7,8] indicates that the entire aromatic environment contains eight tryptophans, eight phenylalanines and nine tyrosine residues whereas the catalytic domain of the molecule, represented by the recombinant product, has the same number of tryptophans but four phenylalanines and seven tyrosine residues.

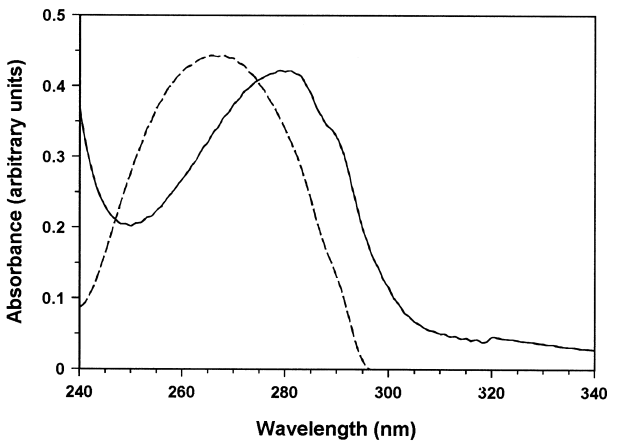

Fig. 1. Protein spectra of endogenous and recombinant cruzipain. The spectrum of endogenous cruzipain is indicated with a continuous line and recombinant cruzipain with a dashed line. The experimental conditions were as described in Section 2.2.

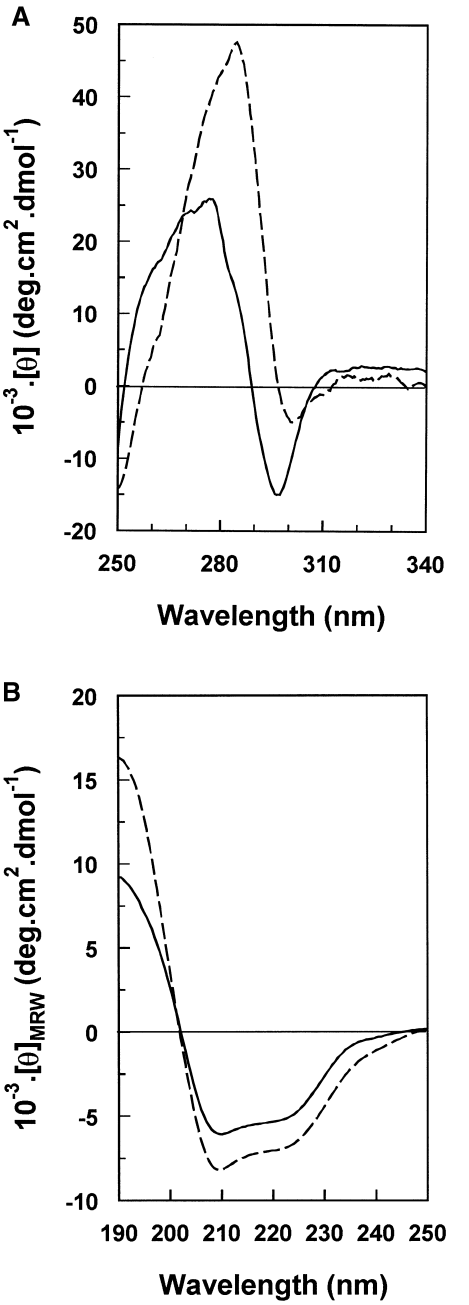

Fig. 2. CD spectra of endogenous and recombinant cruzipain. The experimental conditions for the near-UV (A) and far-UV (B) CD spectra were as described in Section 2.3. The spectrum of endogenous cruzipain is indicated with a continuous line and recombinant cruzipain with a dashed line.

3.2. Circular dichroism

Near-UV CD spectra show that endogenous cruzipain has more fine structure than the recombinant enzyme (Fig. 2A). The spectrum of recombinant cruzipain shows red shifts of 5 nm for the minimum (301 nm vs. 296 nm) and 9 nm for the maximum (285 nm vs. 276 nm), and an almost two-fold higher ellipticity at the maximum wavelength (Fig. 2A). Since the 130 amino acids long C-terminal domain contains six aromatic residues, four phenylalanine and two tyrosine residues and no tryptophan residues [7,8], most probably the observed spectrum reflects a cumulative effect of these con-

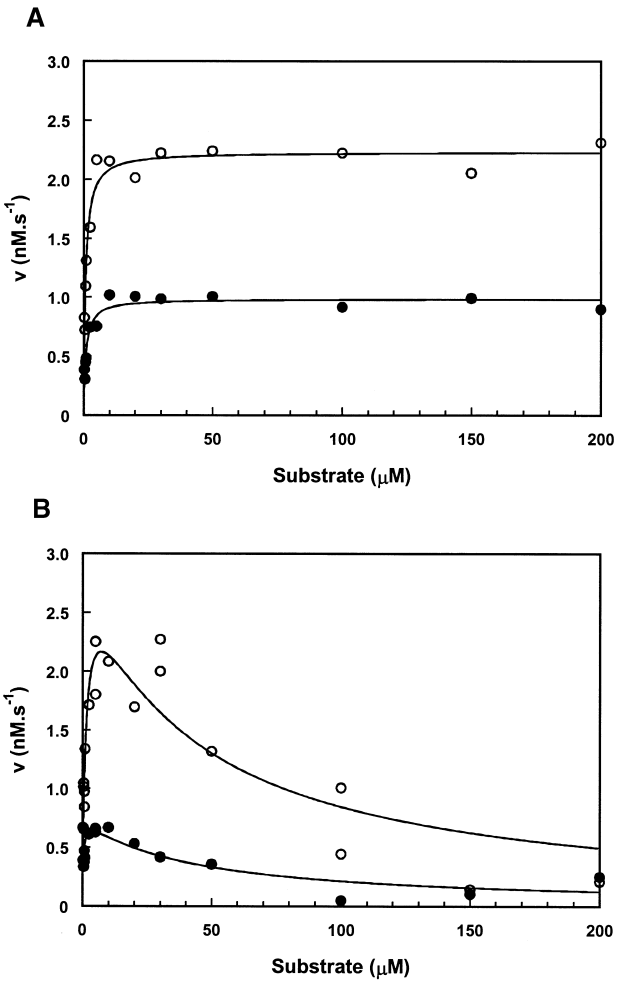

Fig. 3. Dependence of the rate of hydrolysis of cruzipain on substrate concentration at 25°C. A: pH 7.0. B: pH 9.0. The experimental data were fitted at pH 7.0 according to the Michaelis-Menten equation and at pH 9.0 according to the Haldane model [23]. The kinetic parameters for endogenous (●) and recombinant cruzipain (○), respectively, are listed below. The experimental conditions were as described in Section 2.4.

	pH 7.0		pH 9.0	
Parameter	Cruzipain	*r*-Cruzipain	Cruzipain	*r*-Cruzipain
K_m (μM)	0.85 ± 0.15	0.73 ± 0.1	0.38 ± 0.1	1.3 ± 0.5
V_{max} (nM.s^{-1})	0.98 ± 0.03	2.23 ± 0.06	0.75 ± 0.1	2.9 ± 0.4
k_{SI} (μM)	-	-	39 ± 18	41 ± 15

K_m, the Michaelis-Menten constant, V_{max}, maximal velocity, and K_{si} the substrate inhibition constant. *r*−, recombinant.

tributions. The far-UV CD spectrum of endogenous and recombinant cruzipain at pH 7.0 displays a maximum at 190 nm, a negative shoulder at 222 nm and a minimum at 208 nm (Fig. 2B). These profiles suggest that cruzipain belongs to the α+β protein class [19] where α-helix and β-strand secondary structure segments are present in different domains [20]. On the basis of far-UV CD spectra, at least three classes of proteinases within the papain family have been identified, type I (papain, caricain) [21], type II (chymopapain) [21], and type III (bromelain) [22]. The catalytic domain of cruzipain, calculated from the CD spectrum using CONTIN [18], consists of 29% α-helices, 28% β-strand and 42% other elements. Endogenous cruzipain has 19% α-helices, 35% β-strands and 46% of others. Mature endogenous cruzipain consist of both the catalytic domain (215 amino acids) and the C-terminal domain (130 amino acids) [7,8]. Therefore, based on the composition of the secondary structure elements calculated using the CONTIN algorithm [18], and the number of amino acid residues of each domain, we calculated that the C-terminal domain consists of 46% β-structure (60 amino acids) and 54% random coil structure (70 amino acids).

3.3. Dependence of the rate of hydrolysis on substrate concentration and pH

The kinetic parameters V_{max} and K_m were obtained at each pH value by fitting the Michaelis-Menten or Haldane-Wenger models to experimental data in the cases where substrate inhibition was pronounced [23]. Hydrolysis of Z-Phe-Arg-MCA by endogenous and recombinant cruzipain at 25°C in both neutral and acidic pH, followed Michaelis-Menten kinetics (Fig. 3A). However, significant substrate inhibition was observed for both enzyme forms above neutral pH with the effect increasing with increasing pH (Fig. 3B). These results show that substrate inhibition of cruzipain is a pH-dependent process and that the C-terminal domain does not play an essential role in this process. Previous studies on endogenous cruzipain (Y strain) at pH 6.3 indicated a temperature dependence of substrate inhibition [14].

3.4. pH dependence of k_{cat}/K_m

The pH-activity profile of the second order rate constant k_{cat}/K_m allows the determination of the pK_a values, which

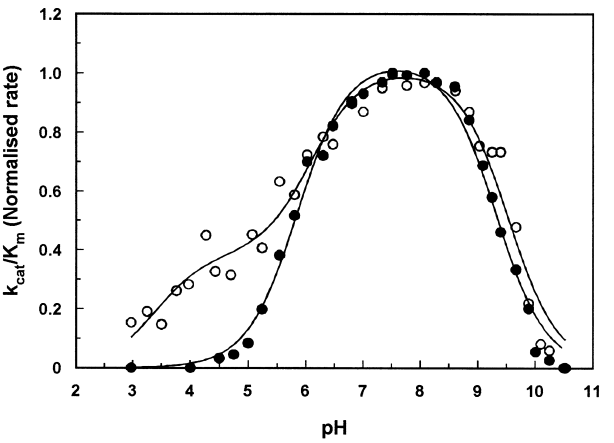

Fig. 4. pH dependence of $(k_{cat}/K_m)_{obs}$ for substrate hydrolysis catalyzed by cruzipain at 25°C. The kinetic parameters for Z-Arg-Arg-MCA (●) and Z-Phe-Arg-MCA (○) are listed below. The experimental conditions were as described in Section 2.5.

Substrate	Z-Phe-Arg-MCA	Z-Arg-Arg-MCA
pK_1	3.4 ± 0.2	
pK_2	6.1 ± 0.2	6.0 ± 0.1
pK_3	9.5 ± 0.1	9.3 ± 0.1
$\bar{k}_1$ x 10^{-6} (M^{-1}s^{-1})	42 ± 5	1.6 ± 0.2
$\bar{k}_2$ x 10^{-6} (M^{-1}s^{-1})	112 ± 35	

The ionization constants (pK_1−pK_3) and pH-independent rate constants (k_1−k_2) were obtained by non-linear regression analysis of the experimental data.

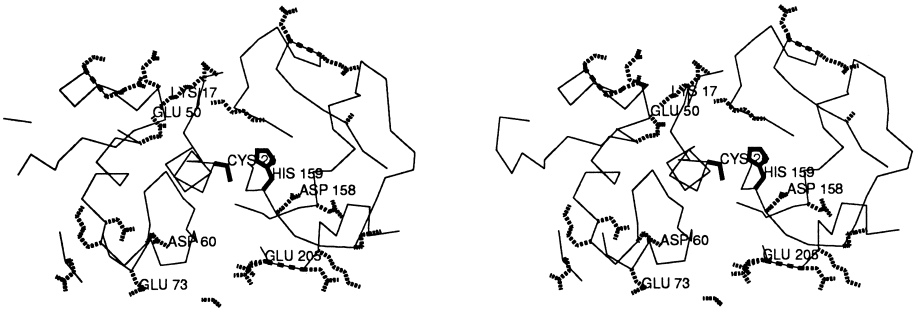

Fig. 5. Top view of the active site region of cruzipain. The residues Cys^{25} and His^{159} (papain numbering) are drawn in bold line. Amino acid residues involved in ion pair formation (Lys^{17}), electrostatic cluster (Glu^{50}) and charged residues in the active site surroundings are denoted.

reflect ionization of the amino acid residues of free enzyme and is not affected by the nature of the rate-limiting step or by the non-productive substrate binding modes [24]. The pH dependence of $(k_{cat}/K_m)_{obs}$ for the hydrolysis of Z-Arg-Arg-MCA by cruzipain showed a bell-shaped profile indicating that at least two ionizing groups with pK_a values 6.0 and 9.3 modulate the enzyme activity (Fig. 4). Hydrolysis of Z-Phe-Arg-MCA by cruzipain was better interpreted by a kinetic model involving three ionizing groups [25] with pK_a values 3.4, 6.1 and 9.5 (Fig. 4). Recent studies on cruzipain specificity with the substrate Abz-LVGGA-EDDnp (where Abz- represents *o*-aminobenzoic acid and EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine) similarly showed three ionizing groups in the enzyme molecule interacting with the substrate with pK_a values 3.59, 6.10 and 8.60 [26]. Lack of the third ionizing group with the lowest pK_a value (3.4) in the case of Z-Arg-Arg-MCA hydrolysis (Fig. 4) could probably be explained by low reactivity of cruzipain towards this substrate at low pH, which is substantially lower than for Z-Phe-Arg-MCA (>40-fold, Fig. 4). These data are also in agreement with previous findings that hydrophobic substrates exhibit broader pH profiles when compared to substrates with more hydrophilic side chains [14,27,28]. The three ionizing groups cannot be identified with certainty, however, there are several charged residues in the vicinity of the active site which are the likely candidates: Asp^{60}, Glu^{73}, Asp^{158}. Glu^{205}, which is located in the S2 position (Fig. 5), has been found to be very important for catalysis [28,29]. Furthermore, Glu^{205} was found to be capable of adopting various conformations, thus enabling cruzipain to bind substrates with both charged and hydrophobic residues in the S2 position [28].

The essential structural features required for the mechanism of action of cysteine proteinases are present in cruzipain (Fig. 5). These include a basic residue at position 17 for ion pair formation and an additional protonic dissociation with a p$K_a \sim 4$ to control the ion pair geometry, most probably due to the Glu^{50} cluster [30]. A sequence alignment of papain-like enzymes shows that those residues are conserved [31]. Nevertheless, site-directed mutagenesis will confirm the specific role of the proposed amino acid residues in the vicinity of the catalytic site motif.

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References

[1] North, M.J. (1982) Microbiol. Rev. 46, 308–340.
[2] Mottram, J.C., North, M.J., Barry, J.D. and Coombs, G.H. (1989) FEBS Lett. 258, 211–215.
[3] Robertson, C.D., Coombs, G.H., North, M.J. and Mottram, J.C. (1996) Perspect. Drug Discovery Design 6, 99–118.
[4] Åslund, L., Henriksson, J., Campetella, O., Frasch, A.C.C., Pettersson, U. and Cazzulo, J.J. (1991) Mol. Biochem. Parasitol. 45, 345–348.
[5] North, M.J. (1991) Parasitol. Today 7, 249.
[6] Watanabe, H., Abe, K., Emori, Y., Hosoyama, H. and Arai, S. (1991) J. Biol. Chem. 266, 16897–16902.
[7] Campetella, O., Henriksson, J., Åslund, L., Frasch, A.C.C., Pettersson, U. and Cazzulo, J.J. (1992) Mol. Biochem. Parasitol. 50, 225–234.
[8] Eakin, A.E., Mills, A.A., Harth, G., McKerrow, J.H. and Craik, C.S. (1992) J. Biol. Chem. 267, 7411–7420.
[9] Souza, A.E., Waugh, S., Coombs, G.H. and Mottram, J.C. (1992) FEBS Lett. 311, 124–127.
[10] Cazzulo, J.J. and Frasch, A.C.C. (1992) FASEB J. 6, 3259–3264.
[11] Del Neri, E., Juliano, M.A., Meldal, M., Svendsen, I., Scharfstein, J., Walmsley, A. and Juliano, L. (1997) Biochem. J. 323, 427–433.
[12] Cazzulo, J.J., Stoka, V. and Turk, V. (1997) Biol. Chem. Hoppe-Seyler 378, 1–10.
[13] Eakin, A.E., McGrath, M.E., McKerrow, J.H., Fletterick, R.J. and Craik, C.S. (1993) J. Biol. Chem. 268, 6115–6118.
[14] Lima, A.P.C.A., Scharfstein, J., Storer, A.C. and Ménard, R. (1992) Mol. Biochem. Parasitol. 56, 335–338.
[15] Stoka, V., Nycander, M., Lenarčič, B., Labriola, C., Cazzulo, J.J., Björk, I. and Turk, V. (1995) FEBS Lett. 370, 101–104.
[16] Turk, B., Križaj, I., Kralj, B., Dolenc, I., Popovič, T., Bieth, J.G. and Turk, V. (1993) J. Biol. Chem. 268, 7323–7329.
[17] Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) Protein Sci. 4, 2411–2423.
[18] Provencher, S.W. (1982) Comput. Phys. Commun. 27, 229–242.
[19] Manavalan, P. and Johnson, W.C. (1983) Nature 305, 831–832.
[20] Levitt, M. and Chothia, C. (1976) Nature 261, 552–558.
[21] Solís-Mendiola, S., Arroyo-Reina, A. and Hernández-Arana, A. (1992) Biochim. Biophys. Acta 1118, 288–292.
[22] Arroyo-Reina, A., Hernández-Arana, A. and Arreguin-Espinosa, R. (1994) Biochem. J. 300, 107–110.
[23] Dixon, M. and Webb, E.C. (1979) Enzymes, Academic Press, New York.
[24] Fersht (1985) Enzyme Structure and Mechanism, Freeman and Company, New York.
[25] Hasnain, S., Hirama, T., Tam, A. and Mort, J.S. (1992) J. Biol. Chem. 267, 4713–4721.
[26] Chagas, J.R., Authié, E., Serveau, C., Lalmanach, G., Juliano, L. and Gauthier, F. (1997) Mol. Biochem. Parasitol. 88, 85–94.
[27] Serveau, C., Lalmanach, G., Juliano, M.A., Scharfstein, J., Juliano, L. and Gauthier, F. (1996) Biochem. J. 313, 951–956.

[28] Gillmor, S.A., Craik, C.S. and Fletterick, R.J. (1997) Protein Sci. 6, 1603–1611.
[29] McGrath, M.E., Eakin, A.E., Engel, J.C., McKerrow, J.H., Craik, C.S. and Fletterick, R.J. (1995) J. Mol. Biol. 247, 251–259.
[30] Pinitglang, S., Watts, A.B., Patel, M., Reid, J.D., Noble, M.A., Gul, S., Bokth, A., Naeem, A., Patel, H., Thomas, E.W., Sreedharan, S.K., Verma, C. and Brocklehurst, K. (1997) Biochemistry 36, 9968–9982.
[31] Turk, D., Gunčar, G., Podobnik, M. and Turk, B. (1998) Biol. Chem. Hoppe-Seyler 379, 137–147.