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Peptide Substrate Specificity of the Membrane-Bound Metalloprotease of *Leishmania*†

Jacques Bouvier,*,‡,§ Pascal Schneider,‡ Robert Etges,‡ and Clément Bordier‡,∥

Institut de Biochimie, Université de Lausanne, CH-1066 Epalinges, Switzerland, Department of Pathology, School of Medicine, University of California, San Francisco, San Francisco, California 94143, and Biokema SA, CH-1023 Crissier-sur-Lausanne, Switzerland

Received February 9, 1990; Revised Manuscript Received June 27, 1990

ABSTRACT: The promastigote surface protease (PSP) of *Leishmania* is a neutral membrane-bound zinc enzyme. The protease has no exopeptidase activity and does not cleave a large selection of substrates with chromogenic and fluorogenic leaving groups at the P_1' site. The substrate specificity of the enzyme was studied by using natural and synthetic peptides of known amino acid sequence. The identification of 11 cleavage sites indicates that the enzyme preferentially cleaves peptides at the amino side when hydrophobic residues are in the P_1' site and basic amino acid residues in the P_2' and P_3' sites. In addition, tyrosine residues are commonly found at the P_1 site. Hydrolysis is not, however, restricted to these residues. These results have allowed the synthesis of a model peptide, H_2N-L-I-A-Y-L-K-K-A-T-COOH, which is cleaved by PSP between the tyrosine and leucine residues with a k_{cat}/K_m ratio of 1.8×10^6 M^{-1} s^{-1}. Furthermore, a synthetic nonapeptide overlapping the last four amino acids of the prosequence and the first five residues of mature PSP was found to be cleaved by the protease at the expected site to release the mature enzyme. This result suggests a possible autocatalytic mechanism for the activation of the protease. Finally, the hydroxamate-derivatized dipeptide Cbz-Tyr-Leu-NHOH was shown to inhibit PSP competitively with a K_I of 17 μM.

Proteolytic enzymes are involved in numerous pathogenic processes, including parasitic disease (Mignatti et al., 1986; Chen & Chen, 1987; McKerrow, 1989). There has been increasing interest in the roles that parasite proteases play in the invasion of host tissues (McKerrow et al., 1989), evasion of the host immune response (Verwaerde et al., 1988), and degradation of host proteins (Rosenthal et al., 1988). These enzymes have therefore been proposed as targets for the rational design of new drugs for chemotherapy, offering an alternative to vaccination (Wang, 1984; Schnebli & Braun, 1986). In this respect, the promastigote surface protease (PSP)[1] of *Leishmania* could be such a target. This enzyme, also known as "gp63", is a glycoprotein expressed at high density (5×10^5 molecules/cell) at the surface of the parasite (Bouvier et al., 1985; Etges et al., 1986a; Bordier, 1987; Chaudhuri & Chang, 1988). It is bound to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Etges et al., 1986b; Bordier et al., 1986) that attaches a wide variety of proteins to membranes (Ferguson & Williams, 1988; Low & Saltiel, 1988). The enzyme is present on the promastigotes residing in the midgut of the phlebotomine sandfly vector (Grimm et al., 1987) and has been detected at the surface of all *Leishmania* species examined so far (Bouvier et al., 1987). Surface metalloprotease activity not only is a highly conserved feature of the genus *Leishmania* but also occurs at the surface of the monogenetic trypanosomatids *Crithidia* and *Herpetomonas* (R. Etges, personal communication). The complete nucleotide sequence for the protease has been deduced (Button & McMaster, 1988, 1990; Miller et al., 1990), and a recent report has shown that the enzyme is encoded by a family of tandemly linked genes, all of which map to a single chromosome (Button et al., 1989). The synthesis and expression of the protease by *Leishmania* amastigotes, the intracellular form infecting the host macrophages, indicate that the enzyme is not stage-specific (Colomer-Gould et al., 1985; Chaudhuri et al., 1989; Medina-Acosta et al., 1989; Frommel et al., 1990). Its involvement in the early phases of infection as a ligand for the mannosylfucosyl receptor, as an acceptor for C3b deposition, or as the major surface antigen and vaccine candidate has been discussed (Russell & Wilhelm, 1986; Mosser & Edelson, 1987; Russell & Alexander, 1988; Puentes et al., 1989). Recently, PSP was shown to be a zinc enzyme, the active site of which has strong similarities to those of other

† This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and from the Swiss National Fund (3-172-0.85).

* Address correspondence to this author at the Department of Pathology, UCSF Medical Center, San Francisco, CA 94143-0506.
‡ Université de Lausanne.
§ University of California, San Francisco.
∥ Biokema SA.

[1] Abbreviations: PSP, promastigote surface protease; Cbz (Z), benzyloxycarbonyl; Suc, *N*-succinyl; AMC, 7-amido-4-methylcoumarin; pNa, *p*-nitroanilide; TBS, Tris-buffered saline; HPLC, high-pressure liquid chromatography.

0006-2960/90/0429-10113$02.50/0

metalloendopeptidases (Bouvier et al., 1989; Jongeneel et al., 1989; Chaudhuri et al., 1989). However, its predominant β-structure, determined by Raman spectroscopy (Jähnig & Etges, 1988) and circular dichroism (Bouvier et al., 1989), distinguishes it from the well-characterized metalloendopeptidase thermolysin (Levitt & Greer, 1977). With the exception of 1,10-phenanthroline, no inhibitors, including α_2-macroglobulin, have any effect on the activity of the membrane-bound enzyme (Etges et al., 1989: Heumann et al., 1989).

To further characterize the *Leishmania* enzyme and to investigate its substrate specificity, natural and synthetic peptides were used as substrates. On the basis of the initial velocity of hydrolysis of several of these substrates, a model peptide was synthesized and used to determine kinetic parameters of PSP. A hydroxamate derivative of a dipeptide was identified as the first potent inhibitor for the *Leishmania* enzyme, effective within the micromolar range. Since PSP is encoded as a pre-pro-enzyme (Button & McMaster, 1988), a synthetic peptide covering the last four amino acid residues of the prosequence and the first five residues of the mature enzyme was used as a potential substrate. This peptide was cleaved by the protease at the amino side of the N-terminal residue of mature PSP, suggesting an autocatalytic activation of the enzyme.

Experimental Procedures

Enzyme and Peptide Substrates. PSP was purified as described earlier (Bouvier et al., 1985). In all the experiments presented in this paper, only the soluble form of PSP (H-PSP) was used, although identical results could be obtained with the amphiphilic form of PSP (unpublished observations). In the first series of experiments, the substrate specificity of the enzyme was investigated by using the following chromogenic *p*-nitroanilide (pNa) and fluorogenic 7-amido-4-methylcoumarin (AMC) substrates: Gly-pNa, Leu-pNa, Leu-AMC, Phe-AMC, Ala-AMC, Gly-Pro-pNa, Cbz-Tyr-pNa, Suc-Leu-Leu-Val-Tyr-AMC, Suc-Phe-Leu-Phe-pNa, Boc-Ala-Ala-pNa, Cbz-Gly-Gly-Leu-pNa, Cbz-Arg-AMC, Cbz-Phe-Arg-AMC, Cbz-Arg-Arg-AMC, Cbz-Val-Leu-Arg-AMC, all purchased from Bachem (Bubendorf, Switzerland). In the second series of experiments, the following natural and synthetic peptides of known amino acid sequence, purchased from Sigma (St. Louis, MO), were used: glucagon; oxidized bovine insulin A and B chains; proctolin (Arg-Tyr-Leu-Pro-Thr); the synthetic peptides Leu-Tyr-Leu, Tyr-Leu, and FAGLA [*N*-[3-(2-furyl)acryloyl]-Gly-Leu-amide]. Peptides 1–25, 39–53, 66–80, and 81–104 (obtained by cleavage with cyanogen bromide) from horse cytochrome *c* (cyt *c*) were received from Dr. G.-P. Corradin (Biochemistry Institute, Lausanne University, Switzerland). Peptides 170–182 from CW-3 HLA antigen (Maryanski et al., 1987) and 83–94 from the β-chain of HLA-DO (Tonnelle et al., 1985) were obtained from Drs. J. Maryanski and R. Accolla (Ludwig Institute for Cancer Research, Lausanne branch, Switzerland). Peptides 94–102 and 94–102 with a serine in position 98 from cyt *c*, as well as the nonapeptide 97–105 from PSP (Button & McMaster, 1988), were synthesized by using the solid-state method of Merrifield modified by Atherton et al. (1979).

Determination of the Peptide Substrate Specificity of PSP. The chromogenic and fluorogenic substrates were stored as 100 mM stocks in dimethyl sulfoxide and used at final concentrations of 100 μM in 200-μL reactions containing 2 nmol of H-PSP. All assays were performed at 37 °C in 10 mM Tris-HCl/140 mM NaCl, pH 7.5 (TBS). Positive controls were specific enzymes added at 2 nmol to the appropriate substrates (e.g., aminopeptidase M with Leu-pNa and Leu-AMC). Chromogenic measurements were made at 405 nm with a Titertek multiscan ELISA reader. Fluorogenic measurements were performed with an Aminco spectrofluorometer by monitoring the increase in the fluorescence emission at 460 nm using an excitation wavelength at 380 nm. Reactions were calibrated with known concentrations of pNa and AMC. All other peptide substrates were incubated at a concentration of 250 μM in TBS with H-PSP at a concentration of 8 nM [molar ratio of enzyme to substrate = $1:3 \times 10^4$]. At times 0, 1, 2, 4, 8, and 16 min after the addition of the enzyme, 20-μL aliquots were removed from the reaction vial, mixed with 10 μL of 0.1 M hydrochloric acid, and heated for 2 min at 95 °C to inactivate the enzyme. Hydrolysis products were resolved by HPLC using a Waters liquid chromatograph equipped with a Waters μ-Bondapak C_{18} reverse-phase column (Waters Associates, Milford, MA) equilibrated with 0.1% (v/v) trifluoroacetic acid in distilled water. From each sample, 15 μL was applied onto the column, and the retained peptides were eluted with a 25-mL, 0–40% linear gradient of acetonitrile, containing 0.1% (v/v) trifluoroacetic acid, at a flow rate of 1 mL/min. The eluate was monitored at 214 nm, and single-elution peaks were collected for subsequent amino acid composition analyses. Peak areas were recorded with a D-2000 Hitachi integrator (Merck, Darmstadt, FRG). The rate of appearance of the products was constant over the first 4–8 min of reaction when less than 20% of the substrate was digested. The initial velocity (v_0) of appearance for each product could therefore be measured in time course experiments. For amino acid analyses, isolated products were freeze-dried and hydrolyzed in 100 μL of constant-boiling 6 M hydrochloric acid (Pierce, Rockford IL), containing 1% (v/v) phenol, for 16 h at 110 °C under vacuum. Samples were then dried under vacuum on sodium hydroxide, dissolved in 30 μL of ethanol/water/triethylamine (2:2:1), dried, derivatized in the dark for 20 min at 25 °C in 80 μL of ethanol/water/triethylamine/phenyl isothiocyanate (7:1:1:1), dried, and dissolved in 200 μL of 50 mM ammonium acetate, pH 6.8. Separation of phenyl isothiocyanate derivatized amino acids was performed as described (Heinrikson & Meredith, 1984). The elution profile was monitored at 254 nm. The relative retention time and the quantitative determination of each amino acid were determined by comparison with known quantities of phenylthiocarbamoyl amino acids run under the same conditions.

Determination of K_m, k_{cat}, and V_{max} for the Synthetic Peptide cyt c 94–102 L. Synthetic peptide 94–102 L of cyt *c* at concentrations of 25, 37.5, 62.5, 125, 250, 750, and 1250 μM was incubated with 8 nM H-PSP in TBS at 37 °C. The initial velocities (v_0) of peptide hydrolysis were determined as described above. These values were then used to determine the K_m, V_{max}, and k_{cat} of PSP for this particular substrate.

pH Stability, pH Optimum, and Inhibition of PSP. The effect of pH on the stability of PSP was determined by incubating the enzyme at a concentration of 130 nM in Britton–Robinson universal buffer (Britton, 1956), at various pH values for 30 min at 37 °C. The v_0 of hydrolysis of the substrate (cyt *c* 94–102 L) was then measured in time course experiments as described above, using Britton–Robinson buffer at pH 7.

The pH optimum of PSP was first determined on azocasein as described by Etges et al. (1986a) with the exception that the buffer system was replaced by the Britton–Robinson universal buffer. The experiment was then repeated using the cyt *c* 94–102 L synthetic peptide as substrate. The v_0 of

hydrolysis was determined, as described above, for each of the designated pH values.

Several compounds containing zinc-coordinating ligands, known to inhibit metalloproteases (Powers & Harper, 1986), were tested with PSP. The hydroxamate derivatives of the amino acid residues leucine and tyrosine (Sigma) and of the dipeptide Z-Tyr-Leu (kindly provided by Dr. E. Shaw, Friedrich Miescher Institute, Basel, Switzerland), as well as the carboxymethyl derivative of the dipeptide Phe-Leu (Sigma), were dissolved in dimethyl sulfoxide to a final concentration of 100 mM. The enzyme was preincubated with a 1 mM sample of each inhibitor for 30 min at 25 °C and then assayed with azocasein as substrate. The determination of the inhibition constant K_I was carried out in time course experiments using different concentrations of azocasein (1, 2, and 4 mg/mL) and concentrations of the inhibitor (0, 10, 50, and 200 μM) with the enzyme at a concentration of 1 μg/mL in a final volume of 2.5 mL of TBS. The reactions were incubated at 37 °C. At times 0, 10, 20, and 30 min, 0.5-mL aliquots were removed and mixed with an equal volume of 5% trichloroacetic acid to stop the reactions. After centrifugation to remove the insoluble material, the concentration of the acid-soluble azocasein peptides was determined spectrophotometrically at 366 nm (Bouvier et al., 1987). The results were then analyzed by using the Lineweaver–Burk and Eadie–Hofstee graphic representations. The inhibition constant K_I was calculated by using the relation $K_I = K_m[\text{inhibitor}]/(K_{m,app} - K_m)$ (Knight, 1986).

Assay of the Ability of PSP to Self-Activate. To test the ability of the protease to self-activate by cleavage of its propeptide, a synthetic peptide covering the V^{100}–V^{101} cleavage site [H_2N-A^{97}-R-S-V-V-R-D-V-N^{105}-COOH; residues numbered according to Button and McMaster (1988)] was synthesized and evaluated as a substrate. The v_0 of hydrolysis of this peptide was determined in a time course experiment as described, with the exception that PSP was used at 80 nM instead of 8 nM. In a separate experiment, the peptide was digested to completion in 1 h under the same conditions, and the products were analyzed to determine the site of cleavage as described for the other peptides.

Results

Peptidase Activity of PSP. In a first series of experiments, several unblocked chromogenic and fluorogenic substrates were used to assay PSP exopeptidase activity. None of these substrates were hydrolyzed by the enzyme. In a second series of experiments, N-terminal-blocked chromogenic and fluorogenic substrates were screened with PSP. Again, none of these substrates were hydrolyzed by the protease. The substrate specificity of PSP was then investigated by using natural and synthetic peptides of known sequence. The enzyme was incubated with each peptide in time course experiments to determine the v_0 of hydrolysis for each product. This approach led to the identification of preferred sites of hydrolysis, indicated by arrows, the width of which is proportional to v_0 (Figure 1). Six peptides (including the propeptide, not presented in Figure 1) were found to be hydrolyzed by the enzyme, resulting in the identification of 11 cleavage sites. Although PSP does not seem to recognize a specific sequence of amino acid residues, it shows some selectivity for basic amino acid residues at the P_2' site,[2] and for hydrophobic residues at the P_1' site. However, polar residues at the P_1' site were also observed (Table I). Although PSP hydrolyzed peptide bonds involving a variety of amino acid residues at the P_1 site, tyrosine residues were observed with high frequency at this particular subsite (Table I). P_2 and P_3' sites can accommodate any class of amino acid residues. Nevertheless, a slight preference was observed for basic residues at the P_3' site. On the basis of these results, two nonapeptides, derived from the cyt *c* 81–104 peptide (cyt *c* 94–102 L and cyt *c* 94–102 S), were synthesized and used as specific substrates for the determination of kinetic parameters. The results show that the substitution of the leucine residue by a serine residue at the P_1' site decreases the v_0 of hydrolysis of the peptide by half (Figure 1, upper part).

K_m, k_{cat}, and V_{max} Determination. On the basis of the high v_0 with which it is cleaved by PSP, the peptide 94–102 L was selected as the most suitable substrate for subsequent kinetic analyses. To determine the K_m, k_{cat}, and V_{max} values of PSP

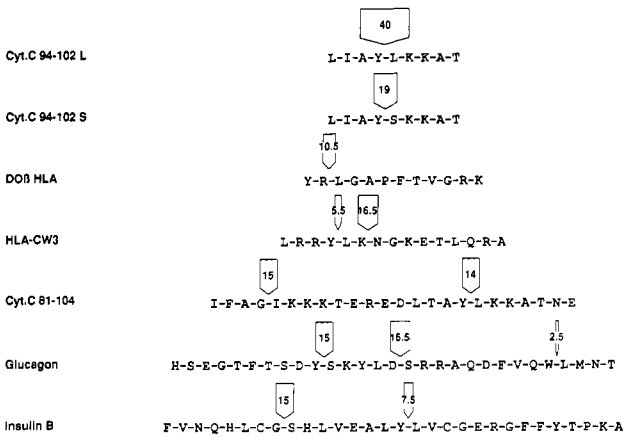

FIGURE 1: Proteolytic activity of PSP on peptides. Time course experiments were performed using a 250 μM sample of each substrate and 8 nM H-PSP at 37 °C in TBS at pH 7.5. Sites of hydrolysis are indicated by arrows, the width of which is proportional to the initial rate of hydrolysis, v_0. The numbers in the arrows represent v_0 and are given in moles of peptide bond cleaved per second per mole of PSP.

Table I: Substrate Specificity of PSP: Frequency of Occurrence of Amino Acid Residues in 11 Cleaved Peptide Bonds[a]

	P_2	P_1	P_1'	P_2'	P_3'
		Acidic			
Asp	1	1	–	–	1
		Basic			
Arg	1	1	–	2	1
His	–	–	–	1	–
Lys	–	1	–	4	3
		Hydrophobic			
Ala	2	–	–	–	1
Ile	–	–	1	–	–
Leu	3	–	5	–	1
Met	–	–	–	1	–
Trp	–	1	–	–	–
Val	–	1	1	1	–
		Polar			
Asn	–	–	1	–	2
Cys^{SO_3}	1	–	–	–	1
Gln	1	–	–	–	–
Gly	–	2	–	2	–
Ser	1	–	3	–	–
Tyr	1	4	–	–	1

[a] The table presents the amino acid residues flanking the scissile peptide bonds shown in Figure 1 (except those of cyt *c* 94–102 L and cyt *c* 94–102 S, which are repetitive) and those of the peptide used in the assay of self-activation of PSP.

[2] The nomenclature of Schechter and Berger (1967) is used to describe the position (P) of the residues in the substrate.

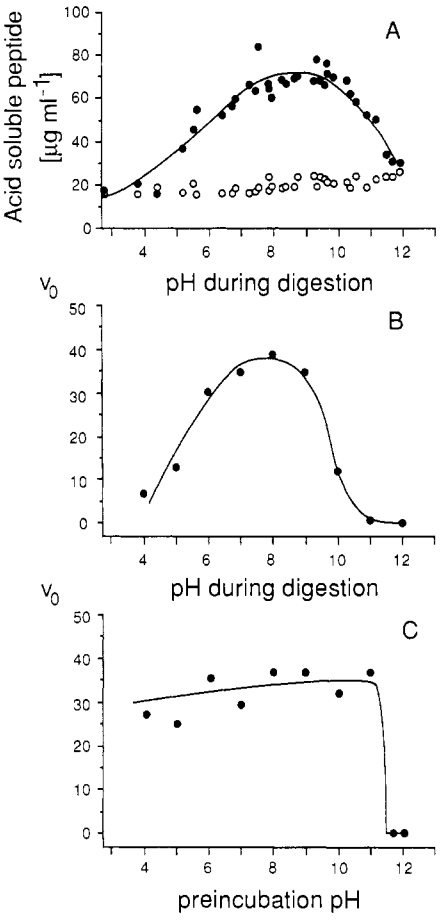

FIGURE 2: Effect of pH on the hydrolysis of azocasein (A) and the synthetic peptide cyt *c* 94–102 L (B) and on the stability of PSP (C). (Panel A) The hydrolysis of azocasein was performed for 30 min at 37 °C, and the concentration of acid-soluble peptides generated was measured spectrophotometrically at 366 nm (closed symbols). For each point, a substrate blank without enzyme was recorded (open symbols) (Panel B) The nonapeptide cyt *c* 94–102 L (250 μM) was incubated at the different pH values with PSP (8 nM) for 30 min at 37 °C. The products of hydrolysis were then analyzed by HPLC as described under Experimental Procedures. (Panel C) The nonapeptide cyt *c* 94–102 L at 250 μM was incubated for 30 min at pH 7, with 8 nM PSP which had been preincubated at the indicated pH values for 30 min at 37 °C. The Britton–Robinson universal buffer was used for both preincubation and assay. In (B) and (C), the v_0 of the reaction was determined in time course experiments and is expressed in moles of peptide cleaved per second per mole of PSP.

for this particular substrate, time course experiments using different substrate concentrations were performed. In each case, the v_0 of hydrolysis of the peptide bond was determined. K_m and V_{max} values were obtained by the graphic analysis of Lineweaver and Burk. PSP cleaves cyt *c* 94–102 L with a $K_m = 2 \times 10^{-2}$ M and a $V_{max} = 40$ mol of peptide bond cleaved per second and per mole of PSP; $k_{cat} = 40\ s^{-1}$. The ratio $k_{cat}/K_m = 1.82 \times 10^6\ s^{-1}\ M^{-1}$.

pH Optimum and pH Stability of PSP. Azocasein was rapidly hydrolyzed by the enzyme over a broad pH range from 7 to 10 (Figure 2A), confirming the results reported previously using different buffers with azocasein and fibrinogen as substrates (Etges et al., 1986a, 1989; Bouvier et al., 1987). Since azocasein is a poor substrate at acidic pH due to its insolubility below pH 5, the pH optimum of PSP was determined using the synthetic peptide cyt *c* 94–102 L as substrate (Figure 2B). The peptide remained soluble throughout the entire pH range. A broad optimum pH, ranging from pH 6 to pH 9, was observed with this substrate. At pH 4, the activity is reduced to 15% of the maximum, to only 2% of the maximum at pH 11, and to undetectable levels at pH 11.5 and 12. To determine the possibility that the low activity of PSP observed at extreme pH values was not due to inactivation of the enzyme, the stability of PSP was determined from pH 4 to pH 12. The enzyme was assayed for activity on cyt *c* 94–102 L at pH 7 following a 30-min preincubation at the indicated pH values (Figure 2C). The enzyme is stable for at least 30 min from pH 4 to pH 11. However, the enzyme is irreversibly inactivated after a 30-min incubation at pH 11.5 and higher.

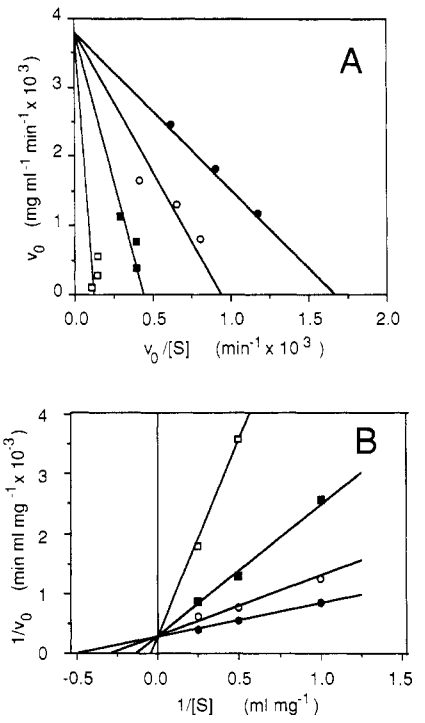

FIGURE 3: Inhibition of PSP by Cbz-Tyr-Leu-NHOH. Eadie–Hofstee (A) and Lineweaver–Burk (B) plots were used to determine the inhibition constant, K_I, using three concentrations of azocasein as substrate and four concentrations of the inhibitor [(●) 0 μM; (○) 10 μM; (■) 50 μM; (□) 200 μM]. The enzyme concentration was kept at 1 μg mL⁻¹. $K_I = K_m[\text{inhibitor}]/(K_{m,app} - K_m) = 17$ μM.

Inhibition of PSP. Several inhibitors known to specifically inactivate metalloproteases were tested for their effects on the activity of PSP. Hydroxamate derivatives of both leucine and tyrosine residues, as well as the carboxymethyl derivative of the dipeptide Phe-Leu, failed to inactivate the enzyme. In contrast, the hydroxamate derivative of the dipeptide Cbz-Tyr-Leu had a dramatic effect on the enzyme. In order to determine the inhibition constant K_I of this compound, four different concentrations of inhibitor and three different concentrations of azocasein were used. The v_0 of hydrolysis of azocasein was determined for each case. The graphic representations of Eadie–Hofstee (A) and Lineweaver–Burk (B) plots of these data are presented in Figure 3. The average K_I value for this new inhibitor is 17 μM.

Potential Self-Activation of PSP. The N-terminal sequence of the purified protein along with the DNA-deduced amino acid sequence of PSP have allowed the identification of the last amino acid residues of the prosequence (Button & McMaster, 1988). On the basis of this information, a synthetic peptide covering the last four amino acid residues of the prosequence and the first five residues of the mature enzyme was used as a substrate. The peptide A-R-S-V-V-R-D-V-N (Figure 4A) was incubated with PSP, and the hydrolysis products were separated by HPLC on a reverse-phase column (Figure 4B). The results of the amino acid analyses performed on the purified peptides are shown above their respective peak

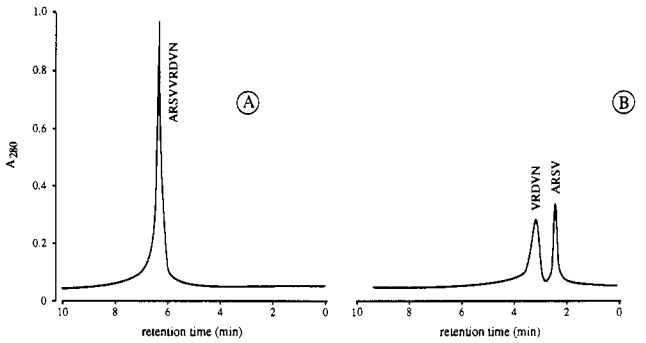

FIGURE 4: Self-activation of PSP. A peptide covering the last four amino acids of the propeptide (A-R-S-V) and the first five residues of mature PSP (V-R-D-V-N) was synthesized and used as a substrate for the enzyme. The purified peptide [250 μM (A)] was incubated with H-PSP (8 nM) in TBS at pH 7.5 for 1 h at 37 °C. The products of enzymatic hydrolysis were separated by reverse-phase HPLC (B) and identified by amino acid analyses, the results of which are indicated for each peak.

(Figure 4). They clearly show that the enzyme cleaves only once and between the two valine residues. The peptide V-R-D-V-N corresponds to the first five residues of the mature enzyme. The v_0 of hydrolysis for this particular peptide bond is $v_0 = 0.46$ mol s^{-1}, indicating that PSP is about 90 times less active on this substrate than on the cyt *c* 94–102 L ($v_0 = 40$ mol s^{-1}).

Discussion

Previous studies on the promastigote surface protease (PSP) of *Leishmania* have led to its identification as a zinc protease (Bouvier et al., 1989; Chaudhuri et al., 1989). The enzyme was shown to hydrolyze protein substrates such as casein, azocasein, gelatin, albumin, hemoglobin, and fibrinogen (Etges et al., 1986a, 1989; Bouvier et al., 1987, 1989; Chaudhuri & Chang, 1988; Chaudhuri et al., 1989). The complexity, large size, and instability at extreme pH values of these substrates precluded their use in the determination of the substrate specificity and pH optimum of the protease. The present study using defined peptide substrates has revealed that *Leishmania* PSP is an endopeptidase with no exopeptidase activity and, consequently, this enzyme can be classified as a member of the EC 3.4.24. subclass.

Like many metalloendopeptidases, PSP shows preference for hydrophobic amino acid residues at the P_1' site (Table I), and its substrate specificity seems to be essentially defined by the P′ subsites of the substrate. However, PSP fails to cleave synthetic substrates designed for thermolysin, and its requirement for hydrophobic residues at the P_1' site is not as strict as those of other metalloproteases (Pozsgay et al., 1986; Mäkinen et al., 1989), as it can accommodate polar residues like serine. Single residues do not alone define the specificity of an enzyme, and the extended binding regions of most endopeptidases have also been shown to play a role. In thermolysin, for example, three residues on the amino side and two residues on the carboxyl side of the scissile bond affect catalysis (Morihara & Tsuzuki, 1970; Kester & Matthews, 1977). The presence of an extended active site for PSP is suggested by the strong similarity between its putative active site and the active site of several metalloendopeptidases (McKerrow, 1987; Bouvier et al., 1989; Jongeneel et al., 1989). In one instance (DOβ-HLA peptide), a dipeptidase activity was observed. Moreover, the fact that the peptide bond Tyr–Leu, which is often cleaved within several of the analyzed peptides, is not always cleaved at the same velocity and, in some cases, not hydrolyzed at all (glucagon) clearly indicates the importance of the amino acid residues flanking the scissile peptide bond. The frequent presence of tyrosine residues at the P_1 site and of basic amino acid residues at the P_2', and to some extent at the P_3' sites of the cleaved peptide bonds, suggests that some specificity exists at these subsites. PSP was also observed to hydrolyze a synthetic substrate covering the last amino acid residues of its prosequence and the first residues of its mature N-terminal sequence at the site expected for the release of the mature form of the enzyme. This event would suggest that an autocatalytic event could account for the activation of the protease. It is of interest to note that the N-terminal amino acid residue of PSP is a valine, followed by an arginine residue. This particular sequence fits the requirement observed in the present study, a hydrophobic residue at the P_1' site and a basic residue at the P_2' site. In addition, Ip et al. (1990) described the specific cleavage by *Leishmania mexicana* PSP of a synthetic peptide with a valine residue in the P_1 site, a threonine (polar) residue in the P_1' site, and lysine residues in both the P_2' and P_3' sites. These findings indicate that the latter two sites contribute to the actual substrate specificity of PSP. The synthetic model peptide, cyt *c* 94–102 L, presenting a tyrosine residue at the P_1 site, a leucine residue at the P_1' site, and two basic amino acid residues at the P_2' and P_3' sites, was hydrolyzed by PSP with a k_{cat}/K_m ratio of 1.8×10^6 s^{-1} M^{-1}. This synthetic substrate was, however, not hydrolyzed by thermolysin, indicating that both metalloenzymes differ with respect to their substrate specificity.

The optimum pH for PSP activity was found to be neutral to basic for *Leishmania major* PSP on a variety of polypeptides (Etges et al., 1986a, 1989); these results are confirmed by using synthetic peptide substrates in this report. Recently, the purified PSP of *L. mexicana* was also shown to have a neutral to alkaline pH optimum (Ip et al., 1990). In contrast, the affinity-purified PSP of *Leishmania amazonensis* was reported to be optimally active at pH 4 (Chaudhuri & Chang, 1988; Chaudhuri et al., 1989). This striking difference may reflect the use of different assays which are not applicable over the extended pH range in which they were used. The "acid" protease of *L. amazonensis* cleaves iodinated BSA optimally at pH 4 with a very low specific activity. In addition, this enzyme was able to digest fibrinogen only at neutral pH (Chaudhuri et al., 1989). In the assays with defined synthetic peptide substrates, where conformational constraints of the substrates are unlikely to interfere with access to susceptible peptide bonds, both the *L. mexicana* (Ip et al., 1990) and *L. major* enzymes show a neutral optimum pH. In the pH stability experiments, we show that the activity of PSP is not irreversibly affected by a 30-min incubation at pH values between 4 and 11 but that at pH 11.5 and higher values enzymatic activity was not recovered upon neutralization.

The identification of a chelating compound as a potent inhibitor for PSP represents an important step in the characterization of the enzyme. This hydroxamate derivative inhibits the protease in the micromolar range, a much smaller concentration than needed for inhibition with 1,10-phenanthroline (millimolar range). However, this new inhibitor is not a substrate analogue. It may inversely fit into the active site of the enzyme, the leucine and tyrosine residues occupying the P_1' and P_2' subsites, respectively, of the enzyme, in order for the hydroxamic moiety to be able to chelate the zinc atom. The observation that the hydroxamic derivatives of the amino acid residues leucine and tyrosine do not inhibit the enzyme, in contrast to the derivative of the dipeptide Z-Tyr-Leu, indicates that at least two residues are required for the inhibitor to fit efficiently into the active site.

The observation that the *Leishmania* enzyme is capable of cleaving a peptide resembling its own propeptide strongly suggests self-activation of the enzyme. PSP would be inactive until it is exposed at the surface of the promastigote, where it could either autocatalytically activate itself or be activated by already active PSP. The low v_0 of cleavage observed for the synthetic propeptide spanning the cleavage site, compared to cyt *c* 94–102 L (87 times slower), would ensure minimal activation of PSP intracellularly. The high density of the enzyme at the surface of the promastigote (5×10^5 per cell) would still permit efficient activation at the surface. An intriguing hypothesis postulates that residues within the propeptide could interact directly with the active-site zinc to ensure the latency of PSP during its biosynthesis. The latency of human fibroblast collagenase is mediated by the interaction of cysteine-73 in the enzyme's prosequence with the active-site zinc (Springman et al., 1990). Interestingly, the pre-propeptide of PSP has two Arg-Cys sites, R-C^{13} in the putative presequence and R-C^{48} in the prosequence, both of which are similar to the R-C^{73} of human fibroblast collagenase (Goldberg et al., 1986), which could complex the active-site zinc of the *Leishmania* enzyme. These hypotheses can be tested only when sufficient quantities of pro-PSP can be isolated.

Although metalloproteases share important features and some similarities for the substrate specificity, they differ widely. There is little, if any, structural similarity between the metalloproteases, except for the highly conserved zinc binding domain. This suggests that the structure of their active sites is the product of convergent evolution and all metalloproteases are likely to use the same basic mechanism for peptide bond cleavage (Vallee & Galdes, 1984; Vallee & Auld, 1990). Virtually every inhibitor of metalloproteases contains a functional group that is able to interact with the zinc atom of the active site. Generally, this group either coordinates or chelates the zinc atom. In addition to the residues involved in catalysis, the active site contains a certain number of residues responsible for substrate recognition which may vary between individual enzymes (Bouvier et al., 1989; Jongeneel et al., 1989), leading to the differences observed for substrate specificity. An important aspect to consider in designing inhibitors is the characterization of the subsite specificity of a given enzyme, in order to define the best peptide sequence to which the zinc ligand will be linked (Chu & Orlowski, 1984; Vencill et al., 1985; Orlowski et al., 1988; Mookhtiar et al., 1988). In this regard, the precise characterization of the substrate specificity of the *Leishmania* surface protease still requires more information. However, the identification of an inhibitor acting within the micromolar range on PSP and the kinetic parameters obtained with the synthetic substrate derived from the cyt *c* peptide constitute the first steps toward the rational design of fluorogenic substrates (Stack & Gray, 1989; Ng & Auld, 1989) and potent inhibitors for the enzyme (Vencill et al., 1985), which will be used to investigate the precise role of the enzyme in the life cycle of the parasite, and eventually serve as model compounds for the chemotherapy of leishmaniases.

ACKNOWLEDGMENTS

We thank Dr. Elliott Shaw from the Friedrich Miescher Institute, Basel, Switzerland, for providing us with the hydroxamate derivative of the dipeptide Z-Tyr-Leu and for his helpful suggestions and Drs. Janet Maryanski and Roberto Accolla from the Ludwig Institute for Cancer Research, Lausanne Branch, Switzerland, and Giampietro Corradin from the Biochemistry Department, University of Lausanne, Switzerland, for providing us with peptides. We also thank Drs. Judy Sakanari, Michael Banda, and Gerry Apodaca from the University of California at San Francisco for their critical reading of the manuscript.

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