Oligopeptidase B from Trypanosoma brucei, a New Member of an Emerging Subgroup of Serine Oligopeptidases*

(Received for publication, February 11, 1999, and in revised form, May 27, 1999)

Rory E. Morty‡‡, John D. Lonsdale-Eccles‡, Jennifer Morehead§, Elisabet V. Caler†, Reinhardt Mentele**, Ennes A. Auerswald**, Theresa H. Coetzter†, Norma W. Andrews‡, and Barbara A. Burleigh‡‡‡

From the ‡Department of Biochemistry, University of Natal, Private Bag X01, 3209 Scottsville, South Africa, the §Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294, the †Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536, and the ‡‡Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik und Poliklinik, Klinikum Innenstadt, Ludwig-Maximilians-Universität München, Nußbaumstraße 20, D 80336 München, Germany

Trypanosoma brucei contains a soluble serine oligopeptidase (OP-Tb) that is released into the host bloodstream during infection, where it has been postulated to participate in the pathogenesis of African trypanosomiasis. Here, we report the identification of a single copy gene encoding the T. brucei oligopeptidase and a homologue from the related trypanosomatid pathogen Leishmania major. The enzymes encoded by these genes belong to an emerging subgroup of the prolyl oligopeptidase family of serine hydrolases, referred to as oligopeptidase B. The trypanosomatid oligopeptidases share 70% amino acid sequence identity with oligopeptidase B from the intracellular pathogen Trypanosoma cruzi, which has a demonstrated role in mammalian host cell signaling and invasion. OP-Tb exhibited no activity toward the prolyl oligopeptidase substrate H-Gly-Pro-7-amido-4-methylcoumarin. Instead, it had activity toward substrates of trypsin-like enzymes, particularly those that have basic amino acids in both P1 and P2 (e.g. benzoyloxy carbonyl-Arg-Arg-7-amido-4-methylcoumarin kcat/Km = 529 s⁻¹ μM⁻¹). The activity of OP-Tb was enhanced by reducing agents and by polyamines, suggesting that these agents may act as in vivo regulators of OP-Tb activity. This study provides the basis for the characterization of a novel subgroup of serine oligopeptidases from kinetoplastid protozoa with potential roles in pathogenesis.

In this study, we identify and characterize a new member of the prolyl oligopeptidase family of serine hydrolases (the “S9” family in the nomenclature of Barrett and Rawlings (1)). This family includes endopeptidases, aminoacylpeptidases, and dipeptidyl aminopeptidases (2). The members of this diverse family share significant amino acid sequence identity within the catalytic domain, and all have activity that is restricted to the hydrolysis of peptides, not proteins (2, 3). Although direct evidence is scarce, proposed roles for these enzymes include neuropeptide and peptide hormone metabolism (4), generation of β-amylloid protein in Alzheimer’s disease (5), memory formation (6), regulation of blood pressure (7), DNA synthesis (8), and processing of the mating pheromone α-factor (9).

As suggested by the name, the majority of the prolyl oligopeptidases cleave their substrates after proline residues. Examples of these enzymes are found in some prokaryotes (10, 11), in yeast (9, 12, 13), and in higher eukaryotes (14–17). However, in some cases, the term prolyl oligopeptidase now appears to be an unfortunate misnomer. This is because a smaller subgroup of this family cleaves substrates on the carboxyl side of basic residues (18–20), not prolyl residues. This subfamily is referred to as oligopeptidase B, e.g. Escherichia coli protease II or oligopeptidase B (EC 3.4.21.83). Until recently, examples of the oligopeptidase B subfamily were restricted to prokaryotes and had received scant attention. However, studies (including this report) have now shown that oligopeptidase B enzymes are also found in trypanosomatids and that these enzymes may play key roles in disease pathology. In the case of the human pathogen Trypanosoma cruzi, the oligopeptidase appears to play a central role in host cell invasion (20, 21). Studies show that oligopeptidase B null mutants of T. cruzi have a markedly impaired ability to infect mice or cultured mammalian cells (21). This impairment seems to be mediated by disruption of the oligopeptidase involvement in trypomastigote-induced intracellular Ca²⁺ transients that occur during mammalian host cell invasion (20, 22). The proposed function of the oligopeptidase in T. cruzi entry is that of a processing enzyme that generates an active signaling ligand for mammalian host cells (23, 24) through the hydrolysis of a stage-specific precursor (20, 21).

However, as with the larger family of prolyl oligopeptidases, it seems likely that more than one function may be ascribed to this enzyme. All life cycle stages of T. cruzi express oligopeptidase B (20), but not all life cycle stages invade mammalian cells. Furthermore, trypsin-like enzymes with properties similar to those of the T. cruzi oligopeptidase B have been described in other kinetoplastids, including the pathogenic parasite Leishmania (Refs. 25 and 26 and this report), and in the African trypanosomes, Trypanosoma brucei, Trypanosoma vivax,
and Trypanosoma congoense (27–29). If these enzymes are homologues of the T. cruzi oligopeptidase B, this raises intriguing questions regarding the roles of these enzymes in parasites with such widely different lifestyles. Thus, one goal of this study was to confirm or refute the contention that the trypsin-like enzymes found in African trypanosomes belong to the oligopeptidase B subfamily of prolyl oligopeptidases.

Oligopeptidase B-like enzymes may be important potential chemotherapeutic targets (as evidenced by the trypanocidal action of many OP-Tb1 inhibitors (30)). Our studies have indicated that the T. brucei oligopeptidase, called OP-Tb, may play an important direct role in the pathogenesis of African trypanosomiasis. During infection, OP-Tb is released into the host bloodstream, where it is insensitive to serum protease inhibitors (28). Hence, it is free to cleave regulatory peptides predicted to be present in host serum. Indeed, the disturbed hormonal pulsatility and endocrine rhythms (31), the unusual cleavage of peptide hormones in the blood of T. brucei-infected rats (32), the diminished levels of regulatory peptides such as atrial natriuretic factor (which is a substrate for OP-Tb (28)) (33), and many of the generalized symptoms of trypanosomiasis (34) all point to the possible role of oligopeptidase B in the disruption of host hormone metabolism during trypanosome infection. Here, we report that, on the basis of gene sequence identity and kinetic analyses, the oligopeptidase from T. brucei (OP-Tb (28)) is an atypical serine peptidase belonging to the oligopeptidase B subgroup of the prolyl oligopeptidase family. Comparison of the deduced amino acid sequences of the trypanosomatid oligopeptidase B genes, including the Leishmania major sequence that we also report here, demonstrates that these enzymes are closely related to the bacterial oligopeptidase B enzymes in terms of sequence identity and substrate specificity. Together, the oligopeptidase B enzymes define a new subgroup of the prolyl oligopeptidase family.

EXPERIMENTAL PROCEDURES

Materials—Fluorogenic peptide substrates were obtained from Sigma, Cambridge Research Biochemicals (Cambridge, United Kingdom), or Enzyme Systems Products (Los Angeles, CA). Peptidyl diazomethane (Sigma, Cambridge Research Biochemicals (Cambridge, United Kingdom), or Enzyme Systems Model 473A gas-phase sequencer following the manufacturer's instructions. Amino acid sequencing of the N termini of OP-Tb was purified as described previously (28), and the concentration (35) and anion-exchange chromatography on DEAE-cellulose separated on a Laemmli SDS-polyacrylamide gel under reducing conditions (42) and blotted onto ImmobilonTM (Millipore Corp., Bedford, MA). Blots were blocked in antibody dilution buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (w/v) nonfat skim milk, 1% (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide) overnight at 4 °C prior to a 1-h incubation at room temperature with 5 mg ml−1 polyclonal anti-T. cruzi recombinant oligopeptidase B IgG (20). Following five 10-min washes in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted to 1:10,000 and developed using the ECL system (Amersham Pharmacia Biotech).

Kinetic Analyses—Substrate specificity of OP-Tb was determined using fluorogenic substrates by preincubation of OP-Tb (1.5 ng, 188 fmol of active enzyme, 5 min) in the same set of assay buffer conditions (45 nM to 75 μM). Km was determined by hyperbolic regression of the kinetic data using the software package Hyper Version 1.01 (obtained from Dr. J. S. Easterby, University of Liverpool, Liverpool, UK). The kcat was determined from kcat = Vmax/[E0], where [E0] represents the active enzyme concentration. The pH profile for OP-Tb was conducted as described above, except that 100 mM ionic strength acetate/Mes/Tris (AMT) buffers (100 mM acetic acid, 200 mM Tris-HCl, 100 mM Mes, 1 mM diethiothreitol, and 4 mM EDTA, I = 0.1) over the pH range 4–12 (43) were used as assay buffer. Similarly, pH stability of OP-Tb was investigated by preincubating OP-Tb (15 ng, 188 fmol of active enzyme, 5 min) in the same set of AMT buffers (37 °C, 1 h) before assaying residual activity of a 10-μl aliquot in AMT buffer at pH 8.

The effect of reducing agents on OP-Tb activity was investigated by preincubating OP-Tb in assay buffer containing diethiothreitol, GSH, or l-cysteine (1–25 mM, 37 °C, 5 min) prior to addition of Cbz-Arg-Arg-AMC (5 μM final concentration). To test for dimerization of OP-Tb under nonreducing conditions, OP-Tb was preincubated in 50 mM Tris-HCl (pH 8) in the absence or presence of diethiothreitol (10 mM) for 5 days at 4 °C. Samples (25 μl, containing 50 ng of OP-Tb) were subsequently reduced by molecular exclusion chromatography on a Sepharhcy S-200 HR column (900 × 15 mm, 0.3 ml min−1, 4 °C) equilibrated in the preincubation buffer. Column fractions were assayed for activity against Cbz-Arg-Arg-AMC as described above. Column fractions collected under nonreducing conditions were reduced prior to assay by preincubating with 10 mM diethiothreitol to reactivate inactive OP-Tb. The effects of divalent metal ions, nucleotides, polyamines, and heparinase B from Trypanosoma brucei

1 The abbreviations used are: OP-Tb, endogenous oligopeptidase B from T. brucei; Cbz, benzoyloxycarbonyl; AMC, 7-amino-4-methylcoumarin; PCR, polymerase chain reaction; Mes, 4-morpholineethanesulfonic acid; Boc, t-butoxycarbonyl.

The full-length T. major oligopeptidase B gene, an L. major LV39 sheared cDNA library (40) was screened in the laboratory of Angela Carle (Universidade de Sao Paulo, Sao Paulo, Brazil) using a 5P-labeled full-length T. cruzi oligopeptidase B gene as a probe. Positive cosmids provided were subcloned into pUC19 following BamHI digestion. Sequence of a positive BamHI subclone revealed that this was a partial clone that lacked an initiation codon. Therefore, sequencing was completed using a positive cosmid clone. 4066 base pairs were sequenced in two segments. Second strand sequencing was carried out to confirm the T. major oligopeptidase B gene sequence (GenBankTM/EBI accession number AF109875).

Generation of Recombinant Oligopeptidase B—The full-length T. brucei oligopeptidase B gene was amplified by PCR from the 5-kilo-base cosmid subclone using primers (forward, 5′-ACTGGGATC-ACCTTCCCCATCAC-3′; and reverse, 5′-CTTAAAGATCCGAATTCTACG-5′) with built-in BamHI sites. PCR was carried out as follows using a Takara LA PCR kit: 94 °C for 2 min; followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 68 °C for 3 min; and a final 10-min extension step at 72 °C. The resulting PCR product was cloned into pCR2.1 (Invitrogen, Madison, WI) and then excised with BamHI to yield a 2.2-kilobase fragment containing the full-length T. brucei oligopeptidase B gene. This fragment was ligated into the expression vector. 150 mM NaCl, 0.05% (w/v) Tween 20, 5% (w/v) nonfat skim milk, 1% (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide) overnight at 4 °C prior to a 1-h incubation at room temperature with 5 mg ml−1 polyclonal anti-T. cruzi recombinant oligopeptidase B IgG (20). Following five 10-min washes in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted to 1:10,000 and developed using the ECL system (Amersham Pharmacia Biotech).
Inhibition of OP-Tb—The mechanism of inhibition of OP-Tb was determined from the effect of inhibitors on the $K_m$ and $V_{max}$ for the hydrolysis of Cbz-Arg-Arg-AMC. For reversible competitive inhibitors, the $K_i$ was determined as described (44). The apparent inhibition constant in the presence of substrate ([S] $\approx$ $K_m$) was given by $K_i$ $=$ $K_{iobs}$ $-$ $K_{iapp}$, where $K_{iobs}$ and $K_{iapp}$ denote substrate concentration. The $K_{iobs}$ was corrected for the presence of substrate by multiplying $K_{iapp}$ by 1 + [S]/$K_m$ (28). The rate constant for complex dissociation ($k_d$) was determined from the relationship $K_i$ $=$ $k_d/k_f$ (45).

RESULTS

Cloning and Sequencing of Oligopeptidase B—The 80-kDa oligopeptidase B (OP-Tb) was purified from $T. brucei$ as described previously (28). Amino-terminal sequence analysis of OP-Tb was unsuccessful, suggesting that the N terminus is blocked. However, four peptides generated by digestion of OP-Tb with endoproteinase Lys-C (Fig. 1) were sequenced and revealed 100% identity to corresponding peptides in the $E. coli$ and $T. cruzi$ oligopeptidase B enzymes. This indicated that the $T. brucei$ oligopeptidase B, as well as a related kinetoplastid protozoan parasite, $L. major$, is related to the oligopeptidase B enzymes of the prolyl oligopeptidase family.

Preliminary Southern blot analysis revealed that the genome of $T. brucei$ as well as a related kinetoplastid protozoan parasite, $L. major$, contained nucleotide sequences homologous to the oligopeptidase B gene of $T. cruzi$ (data not shown). To determine the relationship between OP-Tb and the putative oligopeptidase B homologues detected by Southern blotting, cosmids libraries were screened using the $T. cruzi$ oligopeptidase B gene as a probe. Full-length clones of the oligopeptidase B gene as a probe. Full-length clones of the oligopeptidase B enzyme were obtained. The entire $T. brucei$ oligopeptidase B open reading frame was contained within a 5.0-kilobase $Eco RI/Pst I$ fragment subcloned from a positive cosmid. The gene consisted of 2145 base pairs.
and is predicted to encode a polypeptide of 715 amino acids (Fig. 1). The amino acid sequence of the OP-Tb-derived peptides precisely matched peptide sequences found in the deduced amino acid sequence of the \textit{T. brucei} oligopeptidase B gene (Fig. 1), and we therefore conclude that the \textit{T. brucei} enzyme (OP-Tb) is encoded by the gene for oligopeptidase B. Similarly, the full-length gene encoding the \textit{L. major} oligopeptidase B gene (2196 base pairs) was isolated and sequenced and encodes a similar protein of 732 amino acids (GenBank\textsuperscript{TM}/EBI accession number AF109875) (data not shown). Southern blot analysis using homologous probes revealed that the oligopeptidase B genes of \textit{T. brucei} (Fig. 2) and \textit{L. major} (data not shown) are present as single copy genes per haploid genome, as previously shown for the \textit{T. cruzi} oligopeptidase B gene (20).

The deduced amino acid sequences of the three trypanosomatid oligopeptidase B enzymes exhibited significant homology over their entire sequences. The \textit{T. brucei} oligopeptidase B is 71\% identical to the \textit{T. cruzi} oligopeptidase and 67\% identical to the \textit{L. major} oligopeptidase. The overall similarity of their respective amino acid sequences is 80\%. The similarity of the peptidases was further demonstrated by immunoblot analysis. Polyclonal antibodies generated against the \textit{T. cruzi} oligopeptidase B readily reacted with oligopeptidase B in lysates of \textit{T. brucei} (Fig. 3, lane 4) and \textit{T. cruzi} (lane 5; see also Ref. 20). The full-length \textit{T. brucei} oligopeptidase B gene was expressed in \textit{E. coli} as a catalytically active (Table I), histidine-tagged recombinant enzyme (Fig. 3, lane 1) with a yield of \(-12\) mg/liter of bacterial culture. Immunoblot analysis demonstrated that it was detected at the expected size on a Western blot (Fig. 3, lane 2), similar to the \textit{T. cruzi} recombinant enzyme (last lane 5).

**OP-Tb Substrates**—Consistent with the properties of the prolyl oligopeptidase family (2), our previous findings have demonstrated that OP-Tb is unable to hydrolyze polypeptide substrates (28), including mammalian plasma proteins.\textsuperscript{2} Therefore, fluorogenic peptide substrates were employed for the enzymatic characterization of the native and recombinant forms of the \textit{T. brucei} oligopeptidase B. The reactions followed Michaelis-Menten kinetics. The \(K_m\) values obtained using the recombinant \textit{T. brucei} enzyme approximated those obtained for the purified native enzyme (Table I).

\textsuperscript{2} R. E. M. Morty, unpublished data.
Oligopeptidase B from Trypanosoma brucei

Amidolytic activity of native and recombinant OP-Tb

| Substrate | OP-Tb | rOP-Tb |
|-----------|-------|--------|
|           | \(k_{\text{cat}}/K_m\) | \(k_{\text{cat}}\) | \(K_m\) | \(k_{\text{cat}}/K_m\) | \(k_{\text{cat}}\) | \(K_m\) |
| Cbz-Arg-Arg-AMC | 0.21 | 621.5 | 147.0 | 0.24 | |
| Cbz-Gly-Gly-Arg-AMC | 0.92 | 176.0 | 1.90 | |
| Cbz-Phe-Arg-AMC | 1.12 | 121.0 | 1.00 | |
| Cbz-Ala-Arg-Arg-AMC | 0.21 | 54.4 | 157.0 | 2.91 | |
| Boc-Leu-Lys-Arg-AMC | 0.84 | 42.2 | 76.0 | 1.80 | |
| Boc-Leu-Arg-Arg-AMC | 1.14 | 77.0 | 67.0 | 0.87 | |
| Boc-Gly-Arg-Arg-AMC | 1.87 | 98.3 | 118.0 | 1.22 | |
| Boc-Leu-Gly-Arg-AMC | 1.27 | 39.0 | 87.0 | 2.23 | |
| Boc-Val-Gly-Arg-AMC | 2.21 | 29.3 | 89.0 | 3.03 | |
| H-Ala-Phe-Arg-AMC | 3.13 | 23.1 | 67.0 | 2.81 | |
| Boc-Gly-Lys-Arg-AMC | 3.12 | 24.5 | 55.0 | 2.24 | |
| Boc-Val-Leu-Lys-AMC | 4.04 | 11.1 | 67.0 | 6.06 | |
| Cbz-Arg-AMC | 3.0 | 28.2 | 62.0 | 2.20 | |
| Boc-Ala-Gly-Pro-Arg-AMC | 7.05 | 6.3 | 77.0 | 12.21 | |
| Boc-Val-Pro-Arg-AMC | 9.89 | 5.0 | 62.0 | 12.43 | |
| H-Arg-AMC | 61.6 | 0.34 | 16.6 | 47.1 | |

Amidolytic activity of native and recombinant OP-Tb

| Inhibitor | \(k_b\) | \(t_{1/2}\) | \(k_b\) | \(t\) |
|-----------|---------|---------|---------|--------|
| DCI | 142.10 ± 11.90 | 18 | 196.00 ± 8.01 | 14 |
| AEBSF | 14.00 ± 2.07 | 196 | 15.36 ± 2.21 | 180 |
| DFP | 7.40 ± 0.79 | 375 | ND | ND |
| PMSF | 0.60 ± 0.02 | 4620 | 1.96 ± 0.08 | 1414 |
| pCMFB | 21.90 ± 4.47 | 126 (1854) | 27.60 ± 4.04 | 100 |
| N-Ethylmaleimide | 1.57 ± 0.11 | 1765 (1900) | 1.76 ± 0.19 | 1578 |
| Iodoacetic acid | 1.91 ± 0.08 | 1451 (2559) | 3.59 ± 0.44 | 771 |
| Iodoacetamide | 1.27 ± 0.71 | 2182 (2520) | 1.60 ± 0.27 | 1736 |

**TABLE I**

Irreversible inhibitors of native and recombinant OP-Tb

**TABLE II**

Irreversible inhibitors of native and recombinant OP-Tb

\(a\) No activity was detected against acetyl-Ala-Ala-Pro-Ala-AMC, H-Gly-AMC, H-Leu-AMC, methoxy succinyl-Gly-Tryp-Met-AMC, succinyl-Leu-Tyr-AMC, H-Gly-Pro-AMC, or glutaryl-Gly-Gly-Phc-AMC after 30 min of incubation.

\(b\) rOP-Tb, recombinant histidine-tagged OP-Tb.

\(c\) The S.E. for the \(K_m\) was within 5% of the mean.

\(d\) Assays were conducted in the absence of dithiothreitol. The values in parentheses are the means for reactions in the presence of 10 mM dithiothreitol.

**OP-Tb** Effectors—OP-Tb activity was enhanced by several reducing agents, including dithiothreitol, glutathione, and cysteine. Maximal activation (3-fold) occurred in the presence of 10 mM dithiothreitol (Fig. 4A). This enhancement does not appear to result from the reduction of catalytically inactive disulfide-bonded multimers (as has been demonstrated recently for the thermolysin-like metallo-endopeptidase (soluble metallo-endopeptidase, EC 3.4.24.15) (55)). No significant difference was observed in the elution profiles for two samples of purified OP-Tb fractionated by molecular exclusion chromatography under reducing and nonreducing conditions (Fig. 4B). This suggests that no inactive high molecular mass complexes were formed under our in vitro experimental conditions.

OP-Tb was maximally stable at neutral pH in the absence of dithiothreitol (Fig. 5C). Although OP-Tb had maximal activity at pH 9 (Fig. 5A), it retained considerable activity (75% of maximal activity) at physiological pH (pH 7.4). Over the pH range studied, pH exerted a dramatic effect on the \(k_{\text{cat}}\) (up to 100-fold), whereas the \(K_m\) was relatively unaffected (1.6-fold) (Fig. 5B). The shape of the curve suggests that OP-Tb activity is dependent upon residues with pK\(_a\) values of about 6 and 10. This is consistent with the ionization of active-site histidine and serine residues, respectively, of serine peptidases (56).

The activity of OP-Tb (against Cbz-Arg-Arg-AMC in the presence of 50 mM polyamines) was enhanced by spermine and spermidine (77 and 62%, respectively, over the control values; data not shown). Putrescine and ornithine had no effect. Cursory experiments show that heparin, which carries an opposite charge to polyamines, also enhanced OP-Tb activity (by 58%) at 30 mM polyamine. Neither ATP nor GTP had any effect on the activity of OP-Tb, which is consistent with its being unrelated to the ATP-dependent peptidases.
Benzamidine 254 ND ND
(1.5 ng) was assayed in 100 mM Tris-HCl (pH 8) containing dithiothreitol (●) and spermidine (58) as well as a number of intracellular enzymes, alternate means of controlling its activity must exist. As trypanosomes are known to contain the polyamines spermine and spermidine (58) as well as a number of intracellular reagents such as trypanothione (59), we tested to see if such molecules might regulate the activity of OP-Tb. The activity of OP-Tb was enhanced by reducing agents and by spermine and spermidine. Curiously, dithiothreitol has no enhancing effect on the catalytic activity of the prolyl oligopeptidase from human brain (60) despite its apparent similarity to OP-Tb.

The activity of OP-Tb was inhibited by thiol-reactive agents such as iodoacetate. Because we now know that OP-Tb belongs to the prolyl oligopeptidase group of enzymes, rather than the classic serine protease group, such inhibition is now understandable. It is likely that inhibition by thiol reagents is explained by a crucial cysteine residue (Cys255 in porcine prolyl oligopeptidase and perhaps Cys256 in OP-Tb) that is in close proximity to the catalytic site in the folded enzyme (61). Covalent attachment of bulky thiol-reactive groups to this cysteine residue is predicted to interfere, by steric hindrance, with either the substrate binding or the charge relay system of the catalytic residues.

Prior to the sequencing of the T. brucei and L. major oligopeptidase B genes, the closest homologues of the T. cruzi enzyme were the oligopeptidase B enzymes from E. coli and M. lacunata (20). Here, we find that the trypanosomatid enzymes share ~32% identity with the E. coli and M. lacunata prolyl oligopeptidases, but exhibit less identity to other “true” proline-cleaving enzymes (in the range of 20%). Since sequence homology among members of the prolyl oligopeptidase family is greatest within the catalytic domain (2, 61), this region of the trypanosomatid oligopeptidase B enzymes was aligned with several prolyl oligopeptidases (Fig. 6). The oligopeptidase B enzymes possess the GXXGXXGXX sequence (where X is any residue and Z is a hydrophobic residue) (2) containing the catalytic serine residue (Ser563) in T. brucei (Fig. 1) and

\[
\begin{align*}
\text{Inhibitor} & \quad K_i \quad k_{\text{cat}} \quad k_{\text{cat}/K_i} \\
\text{Leupeptin} & \quad 30.09 \times 10^{-3} \quad 4.76 \times 10^4 \quad 1.43 \times 10^5 \\
\text{Antipain} & \quad 1.58 \times 10^{-3} \quad 1.08 \times 10^6 \quad 1.96 \times 10^5 \\
\text{E-64} & \quad 62.5 \quad \text{ND} \quad \text{ND} \\
\text{Benazamide} & \quad 254 \quad \text{ND} \quad \text{ND}
\end{align*}
\]

\(^a\) Values for the recombinant enzyme were within 5% of those obtained for the native enzyme. No inhibition was observed with amastatin (125 μM), bestatin (125 μM), chicken ovomucoid (100 μg ml−1), chymostatin (1–100 μM), EDTA (1 mM), EGTA (1 mM), elastatinal (150 μM), lima bean trypsin inhibitor (100 μg ml−1), pepstatin (1 μM), 1,10-phenanthroline (1 mM), or soybean trypsin inhibitor (100 μg ml−1). \(^b\) ND, not determined. In these cases, the kcat was too fast to be measured experimentally.

**TABLE III**  
Competitive reversible inhibitors of OP-Tb

**FIG. 4.** Effect of reducing agents on OP-Tb activity. A, OP-Tb (1.5 ng) was assayed in 100 mM Tris-HCl (pH 8) containing dithiothreitol (●), reduced glutathione (○), or l-cysteine (□) at various concentrations. Data points represent the means ± S.E. (n = 3). B, OP-Tb was resolved under reducing or nonreducing conditions by molecular exclusion chromatography on a Sephacryl S-200 HR column (900 × 15 mm, 0.3 ml min−1, 4 °C). Column fractions were assayed for activity against Cbz-Arg-Arg-AMC in the presence of 10 mM dithiothreitol. AFU, arbitrary fluorescence units.

**FIG. 5.** Effect of pH on the activity and stability of OP-Tb. A, OP-Tb (1.5 ng) was assayed in AMT buffers (I = 0.1) over the pH range 4.0–12.0 in the presence of 10 mM dithiothreitol. B, shown are the individual effects of pH on the Kcat (○) and kcat (□) from the data presented in A. C, OP-Tb (10 ng) was incubated for 1 h at 37 °C in AMT buffers (I = 0.1) over the pH range 4.0–12.0 in the absence (□) and presence (●) of 10 mM dithiothreitol. Residual enzymatic activity against Cba-Arg-Arg-AMC was then determined in AMT buffer at pH 8.

**DISCUSSION**

We have previously reported that bloodstream forms of T. brucei possess a high molecular mass trypsin-like serine peptidase (27) and that this enzyme is released into the blood of T. brucei-infected rats (28). The present studies show that this trypsin-like enzyme does not belong to the classic class of trypsins, but instead belongs to the serine peptidases of the prolyl oligopeptidase subgroup. The classic trypsins and chymotripsins all require a free N-terminal amino acid for full expression of enzymatic activity, and yet, abundant activity can be found in OP-Tb, which has a blocked N terminus, and in recombinant OP-Tb, which has an N-terminal polyhistidine tag. Clearly, a free N terminus is not required by this enzyme. Additional differences exist. For example, we see no evidence of a zymogen form (inactive precursor) of OP-Tb. Since each life cycle stage (29) of T. brucei possesses this cytosolic (28, 57) enzyme, alternate means of controlling its activity must exist.
Ser\textsuperscript{577} in \textit{L. major} and exhibit considerable sequence conservation within the catalytic domain. However, even within this highly conserved region, it is clear that the oligopeptidase B subfamily of enzymes (\textit{T. brucei, T. cruzi, L. major, M. lacunata}, and \textit{E. coli}) exhibit greater homology to each other than to the post-proline-cleaving enzymes (Fig. 6). Furthermore, the kinetoplastid (\textit{T. brucei, T. cruzi, and L. major}) oligopeptidase B enzymes are even more similar to each other, exhibiting an overall identity of 70%. Since the oligopeptidase B enzymes can be distinguished from the true prolyl oligopeptidases using sequence identity and substrate specificity as criteria, we propose that the oligopeptidase B enzymes constitute a subfamily of the prolyl oligopeptidase family defined by Barrett and Rawlings (2).

The difference in substrate specificity between the prolyl oligopeptidases and the oligopeptidase B enzymes may be explained by comparing the structure and sequence of the prolyl oligopeptidases with the sequences of the oligopeptidase B enzymes (Fig. 6). Several residues in the active-site pocket are predicted to be involved in substrate recognition by the prolyl oligopeptidases (61). Among these residues is a tryptophan (Trp\textsuperscript{595} in porcine oligopeptidase) that may be involved in stabilizing the interaction with the P\textsubscript{1} proline of the substrate (61). Although this tryptophan residue is conserved in the oligopeptidase B enzymes (Trp\textsuperscript{608} in \textit{T. brucei}) (Fig. 6), it is surrounded by conserved glutamic acid residues in the oligopeptidase B enzymes, but these are absent in the post-proline-cleaving enzymes. Thus, it seems most likely that these negatively charged residues contribute to the recognition of basic substrates by the oligopeptidase B enzymes.

No information regarding the three-dimensional structure of oligopeptidase B is available, although the structure of porcine prolyl oligopeptidase has been reported recently (61). The active sites of prolyl oligopeptidases were proposed to lie buried in active-site “pits” (2). This suggestion is consistent with the structural observation that access of proteins to the catalytic site of porcine prolyl oligopeptidase is likely to be impeded by the positioning of the catalytic apparatus in a tunnel-like cavity (61). This suggestion is supported by our observations reported here and the previously reported observations of others (48) that oligopeptidase B is unable to hydrolyze proteins and that its activity is not inhibited by high molecular mass peptidase inhibitors.

Although the preponderance of known members of the prolyl oligopeptidase family are post-proline-cleaving peptidases, our data indicate that a subgroup of related peptidases is emerging that exhibits specificity for substrates containing paired basic amino acids. To date, these oligopeptidase B enzymes have been identified only in prokaryotes and kinetoplastid protozoan parasites. No oligopeptidase B enzymes have been identified or cloned from mammalian cells. The homologous enzymes from \textit{Saccharomyces cerevisiae} are specific for cleavage after proline residues (9, 13). The preference of oligopeptidase B for cleavage after paired basic residues is intriguing since these sites are abundant in precursors of biologically active molecules and are recognized as sites for processing (62). It was previously suggested that the oligopeptidase B enzymes might function as processing enzymes involved in the generation of biologically active peptides (20, 48). Recently, oligopeptidase B from \textit{T. cruzi} was demonstrated to have a role in the generation of a signaling ligand for mammalian host cells that is involved in the mechanism of host cell invasion by this intracellular parasite.
Oligopeptidase B from Trypanosoma brucei

pathogen (20, 21). Although the physiological function of other oligopeptidase B enzymes is currently unclear, T. brucei OP-Tb may play a major role in pathogenesis of disease through the degradation of regulatory peptide hormones in the blood of infected hosts in African trypanosomiasis (28). Further structural and functional characterization of the oligopeptidase B enzymes in prokaryotes and kinetoplastid protozoan parasites will be useful to better understand the functions this subgroup of the prolyl oligopeptidase family carries out in these organisms and may provide insights into the evolutionary role of this enzyme.

Acknowledgments—Amino acid sequencing work was carried out (by R. M.) in the laboratory of Dr. F. Lottspeich (Max Planck Institute for Biochemistry, Martinsreid, Germany). Screening of the L. major LV39 cosmid library was carried out in the laboratory of Dr. Angela Cruz. We thank Drs. C. Huynh and P. Kima for critical reading of this manuscript.

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