Expression of Recombinant Plasmodium falciparum Subtilisin-like Protease-1 in Insect Cells

CHARACTERIZATION, COMPARISON WITH THE PARASITE PROTEASE, AND HOMOLOGY MODELING*

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Serine proteases play crucial roles in erythrocyte invasion by merozoites of the malaria parasite. Plasmodium falciparum subtilisin-like protease-1 (PfSUB-1) is synthesized during maturation of the intracellular merozoite and accumulates in a set of merozoite secretory organelles, suggesting that it may play a role in host cell invasion or post-invasion events. We describe the production, purification, and characterization of recombinant PfSUB-1 and comparison with the authentic protease detectable in parasite extracts. The recombinant protease requires high levels of calcium for optimum activity and has an alkaline pH optimum. Using a series of decapeptide and protein substrates, PfSUB-1 was found to have a relaxed substrate specificity with regard to the P1 position but is unable to efficiently cleave substrates with a P1 leucine residue. Similarly, replacement of a P4 valine with alanine severely reduced cleavage efficiency, whereas its replacement with lysine abolished cleavage. In all respects investigated, the recombinant protease was indistinguishable from parasite-derived enzyme. Three-dimensional homology modeling of the PfSUB-1 catalytic domain based on an alignment with closely related bacterial subtilisins and an orthologue from the rodent malaria Plasmodium yoelii suggests that the protease has at least three potential calcium ion-binding sites, three intramolecular disulfide bridges, and a single free cysteine within the enzyme S1 pocket. A predicted highly polar S1 pocket and a hydrophobic S4 subsite are in broad agreement with the experimentally determined substrate specificity.

Malaria is caused by protozoan parasites of the genus Plasmodium. In humans, the clinical disease is caused by replication of the blood stages of the parasite. The invasive blood stage form, the merozoite, recognizes and binds a circulating erythrocyte, invades it with the concomitant formation of a parasitophorous vacuole, and then undergoes mitotic replication within this vacuole. Eventual rupture of the host cell releases a new wave of merozoites to repeat the cycle. Erythrocyte invasion and a number of the macromolecular modifications associated with it are sensitive to serine protease inhibitors, and there is a large body of evidence indicating an important role for parasite serine proteases in this critical stage of the parasite life cycle (1). Several putative malarial serine protease genes have been identified, some of which are expressed in the blood stages (1), but in no case is the biological function of the respective gene product known. PfSUB-1 is a Plasmodium falciparum protease belonging to the subtilisin-like superfamily of serine proteases or subtilases (2). The primary structure of the PfSUB-1 putative catalytic domain (MEROPS identification number S08.012; Ref. 3) defines it phylogenetically as probably belonging to a relatively small group of bacterial-like eukaryotic enzymes in the subtilisin or pyrolysin subtilase families (2, 3). PfSUB-1 is synthesized during maturation of the intracellular merozoite and accumulates in dense granules, a group of secretory organelles within the anterior domain of the merozoite (4). During transport to these organelles, the primary PfSUB-1 translation product undergoes two consecutive autocatalytic proteolytic processing steps, both involving cleavage on the C-terminal side of internal aspartic residues; cleavage at the first site converts the PfSUB-1 precursor to p54, and this is in turn converted to p47, the terminal intracellular processing product, upon cleavage at the second site (5). A synthetic decapeptide (Ac-LVSADNIDIS), based on sequence encompassing the p54 processing site, was also found to be cleaved at the Asp-Asn bond by the protease, suggesting that PfSUB-1 has a preference, unusual among subtilases, for cleavage at certain aspartate residues (5). The physiological substrate of PfSUB-1 is unknown. However, its location within secretory organelles of the merozoite makes it ideally situated to play a role in erythrocyte invasion or post-invasion events, and PfSUB-1 is therefore of great interest, both as a potential drug target and because an understanding of its role may shed light on the poorly understood process by which the malaria merozoite gains entry to and modifies its host cell.

Here we describe the expression and purification of recombinant PfSUB-1 and characterization of the protease with respect to its physical and enzymatic properties, inhibitor profile, and substrate specificity. The recombinant enzyme is compared with authentic PfSUB-1 activity detectable in parasite ex...
Recombinant Expression of a Malarial Subtilase

Experimental Procedures

Materials and Anti-PfSUB-1 Antibodies—The baculovirus transfer vector pBacPAK9 was obtained from CLONTECH (Palo Alto, CA). Insect cell media were from Invitrogen and from Expression Systems, LLC (Woodland, CA). Nickel-nitroacetic acid-agarose and nickel-nitroacetic acid Superflow were from Qiagen. All other chromatographic media, the cloning vector pMOSBlue, translation grade [35S]methionine, and molecular mass markers for SDS-PAGE and gel filtration were from Amersham Biosciences. Other biochemicals and protease inhibitors were from Sigma. The production of two different anti-Pf-

Site-directed Mutagenesis—In Vitro Translation—Production of forms of the pfsub-1 gene, in which the coding region is flanked by HindIII and EcoRI restriction sites, was achieved by the method of Stratagene (6) essentially as described previously (5) using the rabbit anti-PfSUB-1m antiserum as described previously (4). A mouse derived anti-PfSUB-1 antiserum, called anti-PfSUB-1m, was raised against a recombinant fusion protein containing the entire PfSUB-1 (4). The region of the PfSUB-1 sequence encompassed by bp54-His6 (residues Asn220-His399) contains 3 tryptophan residues, 29 tyrosine residues, and 7 cysteine residues. Amino acid analysis showed that the latter two classes of amino acids (see “Results”), the predicted molar absorption coefficient at 280 nm in water (ε280 nm) for the bp54-His6 form of rPfSUB-1 is 60,855 M–1 cm–1. Gel Filtration Chromatography—rPfSUB-1 was analyzed by gel filtration on a Superdex 200 HR 10/30 column equilibrated in 50 mM Tris–HCl, pH 8.2, containing 5 mM CHAPS and 150 mM NaCl. The column was calibrated with molecular mass marker proteins ferritin (440 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) in the same buffer. Blue dextran 2000 was used to determine the void volume (V0) of the column. Samples of purified rPfSUB-1 (95 μL) were supplemented with CHAPS to a concentration of 5 mM and applied to the column. The column was eluted at 0.4 mL min–1, and fractions of 0.4 mL were collected. These were then assayed for protease activity as described below.

P1 and P4 Subsite Analysis—The N-acetylated synthetic decapeptide substrate Ac-LVSADNIDIS (PEP1) has been described previously (5). Several variants of PEP1 differing only at either the P1 or the P4 position were produced in-house using standard solid phase Fmoc (9-fluorenylmethoxycarbonyl) chemistry. These were: Ac-LVSADNIDIS (PEP1-D5A), Ac-LVSADNIDIS (PEP1-D5L), Ac-LVSADNIDIS (PEP1-VA5), and Ac-LVSADNIDIS (PEP1-V2A). The variant residues in each case are shown in bold underlined type. All of the structures were confirmed by electrospray mass spectrometry as described previously (5). The peptides were purified by RP-HPLC, and a stock solution of each was prepared in MeSO at a concentration of at least 40 mM. For dilution experiments, dilutions of the stock peptide were first made into MeSO, and then 10 μL of each peptide solution was added to 170 μL of peptide digestion buffer (50 mM Tris–HCl, pH 8.2, 12 mM CaCl2). The mixture was then supplemented with 20 μL of purified rPfSUB-1 (containing ~800 ng of protein). The samples were taken immediately or after incubation at 37 °C, and the reaction was stopped by adding the addition of 3,4-dichloroisocoumarin (DCI, final concentration 80 μM) and freezing on a dry ice/ethanol mix. The samples were analyzed by RP-HPLC on a Vyde 4.6 mm × 25 cm C18 reversed-phase column and eluted at 1.0 mL min–1 with a 9–58.5% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid over 60 min. For apparent Km determinations, preliminary time courses of peptide digestion were first carried out to optimize the digestion conditions and to define the period over which the hydrolysis proceeded at a constant rate; the reaction times eventually chosen for the Km(app) determinations ranged from 15 to 120 min. Peptides at a range of concentrations were incubated with a standard amount of rPfSUB-1 at 37 °C, and the reactions were stopped as described above. Aliquots of digests were analyzed by a rapid RP-HPLC method on the Vyde 4.6 mm × 25 cm C18 reversed-phase column and eluted at 1.0 mL min–1 with a 27% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid over 10 min. Elution was monitored by measuring absorbance at 215 nm. All of the kinetic determinations were based on production of the common C-terminal product of peptide cleavage, NH2-NIDIS-Oh, which elutes with a retention time of ~8.9 min under these conditions. The relationship between peak height and absolute quantity of this peptide was determined in preliminary experiments by RP-HPLC analysis of known sintered glass funnel, and washed with a total of 1.2 liters of ice-cold phosphate-buffered saline, pH 7.2. The bound proteins were eluted with 2–3 gel volumes of 2 M NaCl in 50 mM phosphate buffer, pH 8.2. The eluate was supplemented with imidazole to 1 mM, adjusted to pH 8.2, and then loaded onto a 6-ml column of nickel-nitroacetic acid Superflow. The column was washed with 200 µL of 50 mM NaCl in 50 mM phosphate buffer, pH 8.2, containing 300 mM NaCl, then washed with 100 µL of 25 mM imidazole in the same buffer, and then eluted with 250 mM imidazole in 20 mM Tris–HCl, 300 mM NaCl, pH 7.6. The eluted protein peak was pooled, supplemented with two volumes of 20 mM Tris–HCl, pH 7.6 to adjust the NaCl concentration to 100 mM, and then loaded onto a pre-equilibrated column of Q-Sepharose Fast Flow. The column was washed with 20 mM Tris–HCl, 150 mM NaCl, pH 7.6, and then eluted with a 30-mL gradient of 150–400 mM NaCl in the same buffer. The rPfSUB-1 peak was pooled, dialyzed exhaustively against 20 mM Tris–HCl, pH 7.6 at 4 °C, supplemented with glycerol to 10% (v/v), and stored in aliquots at −70 °C. Yields of pure product were estimated using the predictive method described by Pace et al. (8), which is based on the number of tryptophan, tyrosine, and cysteine residues within the protein. The recombinant product is produced mostly in the form of its 54-kDa processed product, previously referred to as bp54-His6 (5). The region of the PfSUB-1 sequence encompassed by bp54-His6 (residues Asn220–His399) contains 3 tryptophan residues, 29 tyrosine residues, and 7 cysteine residues. Approximately 50% of the latter two classes of amino acids (see “Results”), the predicted molar absorption coefficient at 280 nm in water (ε280 nm) for the bp54-His6 form of rPfSUB-1 is 60,855 M–1 cm–1. Gel Filtration Chromatography—rPfSUB-1 was analyzed by gel filtration on a Superdex 200 HR 10/30 column equilibrated in 50 mM Tris–HCl, pH 8.2, containing 5 mM CHAPS and 150 mM NaCl. The column was calibrated with molecular mass marker proteins ferritin (440 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) in the same buffer. Blue dextran 2000 was used to determine the void volume (V0) of the column. 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amounts of PEP1 that had been digested with trypsin digestion buffer, supplemented with the various peptide substrates (final concentration, 2 mM) or pepF1-6R (final concentration, 20 mM), and then incubated at 37 °C for up to 4 h for digestion to proceed. Aliquots (100 μl) of digestion supernatant were analyzed by RP-HPLC as described above.

For immunodepletion experiments, a preparation of the Triton X-100-insoluble schizont fraction was further extracted by sonication into 5 volumes of peptide digestion buffer supplemented with 5 mM CHAPS and 1 mM NaCl. This was found to effectively solubilize the PEP1-hydrolyzing activity. The CHAPS extract was clarified by centrifugation (12,000 × g for 15 min), divided into aliquots on ice, and stored at −70 °C until used. Scintillation proximity assay was performed with the radiolabeled protein-G-Sepharose beads that had been preadsorbed with saturating amounts of anti-PSUB-1 serum or preimmune serum, and then washed in peptide digestion buffer before use. Following a 1-h incubation with frequent vortexing, the protein-G-Sepharose beads were removed by centrifugation, and the treated schizont extracts were assayed for the presence of PEP1-hydrolyzing activity as described above.

Sequence Alignment and Homology Modeling—Homology searches of the incomplete P. falciparum and other Plasmodium genome data bases were performed with the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (National Institutes for Health) using the CustomBLAST server at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/blast/). Preliminary sequence data from the Plasmodium yoelii genome was obtained from The Institute for Genome Research website (www.tigr.org/db/edh2/pya1/htmls/). This sequencing program was carried on in collaboration with the Naval Medical Research Center and is supported by the U.S. Department of Defense. A single continuous P. yoelii open reading frame encoding a protein with significant homology to PSUB-1 was identified by a tblastn search, querying with the sequence encompassing His430–Ala615 of PSUB-1. The identified open reading frame contained a single unassigned base at position 683. The identity of both this and the surrounding nucleotide sequence was confirmed by PCR amplification of a 336-bp DNA fragment from P. yoelii clone YM genomic DNA (a kind gift of Dr P. Freiser, National Institute for Medical Research, U.K.) using primers 5′-TAGAATTAATTTGAGCATATGAGAAATTAGAA-3′ (sense) and 5′-TTATCATTTCTTGAGCATCATCTAAACGG-3′ (antisense). The PCR product was cloned into pMOSBlue and completely sequenced on both strands. Construction of a three-dimensional structural model of the catalytic domain of PSUB-1 was based on a sequence alignment with four closely homologous prokaryotic subtilases whose structures have been determined by x-ray crystallography. The coordinates were obtained from the Protein Data Bank (14). For sequence alignment, secondary structure was assigned using DSSP (15) or predicted (for the malarial subtilases) using PHD (16). Modeling was performed using QUANTA (Release 4.0, Accelrys) running on a Silicon Graphics INDIGO2 work station under the UNIX operating system. Energy minimization was accomplished using the CHARMM force field (17).

RESULTS

Protein Expression and Purification—Design of the psub1-synth gene was originally based on the preferred codon usage of P. pastoris with the intention of using this organism for high level expression of the protease. Unfortunately, when expressed in Pichia the gene product remained intracellular in an insoluble, full-length, presumably aggregated form (6). Expression of the same gene in recombinant baculovirus-infected High Five insect cells resulted in secretion of a recombinant product detectable by Western blot with PSUB-1–specific antibodies. As previously reported (5), secretion of correctly processed PSUB-1 substrate was hydrolyzed the presence of low levels of tunicamycin, because in the absence of the drug, PSUB-1 was secreted in a high molecular mass unprocessed form, perhaps as a result of inappropriate N-linked glycosylation of the predicted oxoyanone hole residue Asn522. In common with a number of blood stage P. falciparum proteins (18–20), PSUB-1 appears not to be subject to N-glycosylation in the parasite (5). Preliminary trials showed that the secreted recombinant protein could be readily recovered from insect cell culture supernatants by binding in “batch” to the Cibacron Blue F3G-A-
The protein yields were calculated using the predictive absorption method described by Pace et al. (8). Despite the uncertainty regarding the stoichiometry of the interaction between bp54-His$_6$ and the bp31 species in the purified preparations, this method was considered to provide a reasonable means of estimating yields of bp54-His$_6$, because bp31 (which extends from Lys$^{286}$ to Asp$^{219}$, Ref. 5) contains no tryptophans and only a single tyrosine residue. Its contribution to the absorption of the purified complex at 280 nm is therefore likely to be negligible. Based on the calculated $e_{280}$ of 60,085 M$^{-1}$ cm$^{-1}$ and a predicted molecular mass of 53,809 Da, the final yields of purified bp54-His$_6$ were estimated to be in the order of 2–5 mg/liter of starting culture supernatant. For convenience the preparations are usually henceforth referred to simply as rPfSUB-1.

**Physical Features of Purified rPfSUB-1**—The UV absorption spectrum of the purified rPfSUB-1 displayed pronounced absorption above 315 nm (not shown), suggestive of substantial light scattering caused by protein aggregation. This suspicion was confirmed by dynamic light scattering analysis, which showed the preparations to be highly polydisperse; consistent with this, attempts to subject the purified material to native polyacrylamide gel electrophoresis in the absence of detergent were unsuccessful, the bulk of the material apparently being unable to enter the resolving gel (not shown). Despite this, extensive but unsuccessful attempts were made to crystallize the product following treatment with para-hydroxymercuribenzoate (pHMB), a potent inhibitor of PfSUB-1 protease activity (5), and with or without various detergents. The purified protease also displayed an unexpected tendency to adsorb tightly and apparently nonspecifically to a variety of matrices. These included various chromatographic media and the interior of quartz cuvettes. This characteristic precluded a number of approaches that might otherwise have been useful for further physical characterization of the product. For example, attempts to fractionate the pure material on a Superdex 200 HR 10/30 gel filtration column in the absence of detergent were reproducibly unsuccessful. Under these conditions all of the material applied to the column bound strongly to the matrix and was not eluted (not shown). Gel filtration in the presence of the zwitterionic detergent CHAPS was more informative (Fig. 1B). Under these conditions ~10% of the applied protease activity was eluted. Although it did so in a very broad peak, suggesting either substantial mass heterogeneity or physical interactions with the column, the peak of eluted activity migrated with an apparent molecular mass of 56.7 kDa, very close to the predicted mass of 54 kDa for the bp54-His$_6$ component and substantially below that of the predicted mass of about 80 kDa for an intact bp31/bp54-His$_6$ complex (5). Our data indicate that the major enzymatically active component of the recombinant material exists in the form of free, monomeric bp54-His$_6$.

**pH and Cation Dependence and Inhibitor Profile of PfSUB-1**—Kinetic experiments using the fluorescent substrate pepF1-6R were used to explore the cation dependence and the pH optimum of rPfSUB-1 activity. Fig. 2A shows that enzyme activity was dependent on the presence of substantial amounts of added calcium, with maximal activity only reached at a calcium concentration of 8–12 mM; the rate of pepF1-6R hydrolysis in 12 mM calcium was ~3-fold greater than that in the absence of added calcium. The effect was calcium-specific, because the addition instead of MgCl$_2$ or NaCl at concentrations of up to 16 mM had little or no activating effect on enzyme.

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activity, and indeed at higher concentrations both were slightly inhibitory. Similarly, the addition of excess amounts of the calcium chelator EGTA or EDTA reduced protease activity down to levels similar to that observed in the absence of added calcium (Table I). In further experiments (Table I) it was found that rPfSUB-1 activity was only moderately sensitive to the serine protease inhibitors chymostatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and 4-(2-aminoethyl)benzenesulfon- 

yl fluoride but was potently inhibited by DCI. The protease was also exquisitely sensitive to pHMB, confirming previous nonquantitative data (5), but was relatively unaffected by a different alkylation agent, iodoacetamide. The presence of 10 mM l-cysteine had only a slight effect on rPfSUB-1 activity, whereas the more potent reductant dithiothreitol was strongly inhibitory.

Fig. 2B shows that the recombinant enzyme exhibited a relatively alkaline pH optimum, with maximal activity at about pH 8.1. Activity dropped sharply below about pH 7.5; it is likely that this is directly due to a reduction in enzyme activity rather than markedly reduced protease stability at lower pH, because the insect culture supernatant from which the rPfSUB-1 had been originally purified was pH 6.0.

Analysis of the Substrate Specificity of PfSUB-1—In subtilases, at least four binding sites in the active site groove of the enzyme are involved in the interaction with substrate. Substrate specificity is generally primarily mediated by interactions with the P4-P1 residue side chains in the large S1 and S4 binding pockets (2, 21–23). The peptide substrate PEP1 (Ac-LVSA-DNIDIS) is efficiently cleaved at the Asp-Asn bond by rPfSUB-1 (5). To establish the contribution of the P1 Asp to the specificity of PEP1 cleavage by PfSUB-1, six variants of PEP1 were produced, differing only at the P1 or P4 position, and assessed for their susceptibility to cleavage by purified rPfSUB-1. As shown in Fig. 3A, three of the P1 variant peptides, (PEP1-D5E, PEP1-D5N, and PEP1-D5A) were cleaved with varying degrees of efficiency. In contrast, the P1 Leu variant PEP1-D5L was completely resistant to cleavage and remained so even after extended incubations of up to 8 h (not shown). Note that in every case where cleavage did occur it resulted in the appearance of only two products, one of which (with a retention time of 11.5 min under these conditions) was common to all of the digests. This has previously been shown to correspond to NH2-NIDIS-OH, the C-terminal product of cleavage at the predicted site (5). Because of the limited solubility of the PEP1-D5N variant, it was not possible to determine the kinetic parameters for its hydrolysis by rPfSUB-1. However, this was achieved for PEP1 and the other peptides (Fig. 3B). Table II summarizes these data and shows that the V_max/K_m(app) values (a measure of enzymatic specificity; Ref. 24) for both PEP1-D5E and PEP1-D5A were approximately half that of PEP1 (observed value, 0.76 h⁻¹). The major contributing factor to this difference was that V_max for PEP1 was over 2-fold higher than for either PEP1-D5E or PEP1-D5A in the presence of identical amounts of enzyme, indicating that under conditions of substrate saturation, the overall turnover rate of this parental substrate is substantially higher than any of the variants. These results indicate that although PfSUB-1 has a rather relaxed requirement with respect to the P1 subsite, it prefers Asp to Ala or the longer side chain of Glu in the P1 position, and it cannot cleave substrates with a P1 Leu.

Furthermore, the enzyme displayed no significant capacity to cleave any of the tested peptides at sites other than the major

| Inhibitor Profile of PfSUB-1 |
|-----------------------------|
| Concentration (mM) | PfSUB-1 activity (%) |
|---------------------|----------------------|
| Control             | 100                  |
| L-Cysteine          | 10.0                 |
| Dithiothreitol      | 10.0                 |
| PMSF (0.5)          | 2.0                  |
| AEBSF (3 μM)        | 2.0                  |
| DCI (0.08)          | 0.08                 |
| Chymostatin (20 μM) | 20.0                 |
| Leupeptin (20 μM)   | 20.0                 |
| pHMB (1 M)          | 1.0                  |
| Iodoacetamide (10 μM)| 10.0                 |
| EDTA (10 μM)        | 10.0                 |
| EGTA (10 μM)        | 10.0                 |

TABLE I

The effects of selected compounds on the hydrolysis of fluorescent substrate pepF1-6R by rPfSUB-1 were determined as described under "Experimental Procedures." The rates were determined following incubation of the inhibitors at the stated concentration with enzyme for 5 min at 37 °C.

The values shown are the means of duplicate measurements (variation is <4%).

Concentration in μg ml⁻¹. AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.
purified protease preparations. However, assuming that 100% of the recombinant PfSUB-1 is catalytically active (which is unlikely given the presence of substantial amounts of co-purifying pro-domain species, as discussed above), this represents a \( k_{\text{cat}} \) of \(-2.2 \text{ s}^{-1}\), which compares reasonably favorably with \( k_{\text{cat}} \) estimates for cleavage of various optimal peptide substrates by other members of the subtilisin superfamily, including furin \((k_{\text{cat}} = 11–29.3 \text{ s}^{-1}; \text{Ref. 25}),\) Kex2 protease \((k_{\text{cat}} = 50 \text{ s}^{-1}; \text{Ref. 26}),\) and subtilisin BPN’ \((k_{\text{cat}} = 41–50 \text{ s}^{-1}; \text{Refs. 27 and 28}).\)

To confirm the above conclusions regarding PfSUB-1 substrate specificity in the context of a polypeptide substrate, advantage was taken of our previous observation \((5)\) that in vitro translated PfSUB-1 possesses no enzymatic activity but can be correctly processed in trans at the authentic p54 cleavage site \((\text{Asp}^{110–\text{Asn}^{120}})\) by baculovirus-derived rPfSUB-1. The \(\text{pfsub-1}^{\text{synth}}\) gene or three mutant forms of it in which the catalytic, because mutation of the predicted active site serine to an Ala residue completely abrogates processing site. Under the conditions of the assay, the calculated \(V_{\text{cat}}\) of \(585 \pm 29 \text{ µM h}^{-1}\) for PfSUB-1 was only poorly cleaved, whereas PfSUB-1 and PfSUB-1-D5L were not cleaved at all. Minor peaks evident in the elution profiles of PfSUB-1-D5A, PfSUB-1-D5L, and PfSUB-1-V2A are due to contaminating species in the starting peptide preparations. Peptide elution profiles are shown from 5 to 40 min following sample injection, and data analysis were as described under “Experimental Procedures.”

| Peptide          | \(k_{\text{cat(app)}}\) | \(V_{\text{max}}\) | Relative \(V_{\text{pool}}/k_{\text{cat(app)}}\) |
|------------------|-------------------------|------------------|---------------------------------|
| PfSUB-1          | 772 \pm 92              | 585 \pm 29       | 1.00                            |
| PfSUB-1-D5E      | 769 \pm 184             | 245 \pm 22       | 0.42                            |
| PfSUB-1-D5A      | 436 \pm 54              | 190 \pm 8        | 0.58                            |
| PfSUB-1-D5N      | ND                      | ND               | ND                              |
| PfSUB-1-D5L      | ND                      | ND               | ND                              |
| PfSUB-1-V2A      | 2063 \pm 340            | 69 \pm 5         | 0.04                            |
| PfSUB-1-V2R      | ND                      | ND               | ND                              |

The \(V_{\text{cat}}/K_{\text{m}}\) values are shown relative to that for cleavage of PfSUB-1 (actual value was 0.76 \text{ h}^{-1}\) under the conditions used.

**FIG. 3** Cleavage of PfSUB-1 by PfSUB-1. A, peptide substrate PfSUB-1 and several variants possessing substitutions of the P4 Val or P1 Asp residue were dissolved at a concentration of \(-500 \text{ µM}\) in peptide digestion buffer, and then 180-µl samples were supplemented with 800 ng of purified PfSUB-1. Aliquots of equal volume were taken immediately upon addition of protease \(\text{(upper trace in each panel)}\) or following incubation at 37°C for up to 4 h \(\text{(lower trace in each panel)}\) and analyzed by RP-HPLC. The HPLC elution profiles are shown from 5 to 40 min following sample injection, depending on the retention properties of the parent peptide. Peptide PfSUB-1-V2A was only poorly cleaved, whereas PfSUB-1-D5L and PfSUB-1-V2K \((\text{not shown)}\) were not cleaved at all. Minor peaks evident in the elution profiles of PfSUB-1-D5A, PfSUB-1-D5L, and PfSUB-1-V2A are due to contaminating species in the starting peptide preparations. B, representative plot of the initial rate of cleavage versus substrate concentration for selected substrates \(\text{(PfSUB-1 and PfSUB-1-D5A). Curve fitting was by nonlinear regression using GraFit 5 software. Each curve is fitted to the accumulated results of three independent assays. The enzyme assays and data analysis were as described under “Experimental Procedures.”}

TABLE II

Kinetic parameters for cleavage of PfSUB-1 and PfSUB-1 variants by PfSUB-1

| Peptide  | \(k_{\text{cat(app)}}\) | \(V_{\text{max}}\) | Relative \(V_{\text{pool}}/k_{\text{cat(app)}}\) |
|----------|-------------------------|------------------|---------------------------------|
| PfSUB-1  | 772 \pm 92              | 585 \pm 29       | 1.00                            |
| PfSUB-1-D5E | 769 \pm 184             | 245 \pm 22       | 0.42                            |
| PfSUB-1-D5A | 436 \pm 54              | 190 \pm 8        | 0.58                            |
| PfSUB-1-D5N | ND                      | ND               | ND                              |
| PfSUB-1-D5L | ND                      | ND               | ND                              |
| PfSUB-1-V2A | 2063 \pm 340            | 69 \pm 5         | 0.04                            |
| PfSUB-1-V2R | ND                      | ND               | ND                              |

The \(V_{\text{cat}}/K_{\text{m}}\) values are shown relative to that for cleavage of PfSUB-1 (actual value was 0.76 \text{ h}^{-1}\) under the conditions used.
capacity to cleave a Leu-Asn bond.

To determine the importance of the P4 residue in substrate recognition by PSIUB-1, two further derivatives of PEP1 called PEP1-V2K (Ac-LKSADNIDIS) and PEP1-V2A (Ac-LÅSAĐNIDIS) were assessed for susceptibility to digestion by the protease. Prolonged incubation of PEP1-V2K with rPSUB-1 resulted in no detectable cleavage (not shown). Prolonged incubation of PEP1-V2A with rPSUB-1 resulted in only minimal cleavage of this peptide (Fig. 3A), and these were reflected in the kinetic measurements (Table II), which indicated that this variant is a very poor substrate for the enzyme. Our data suggest that the P4 residue plays an important role in substrate recognition by PSIUB-1.

**Detection of Endogenous PSIUB-1 Activity in Malaria Parasite Extracts**—Previous work has shown that PSIUB-1 is synthesized during the latter stages of merozoite maturation in the intraerythrocytic schizont stages of *P. falciparum*. The protein can be readily biosynthetically radiolabeled in mature schizonts and can be detected by immunoprecipitation or Western blot analysis of detergent extracts of both schizonts and naturally released merozoites (4, 5). The only previous direct demonstration of PSIUB-1-derived protease activity in parasites made use of radiolabeled, *in vitro* translated PSIUB-1 itself as a substrate and suggested that the endogenous PSIUB-1 activity resided in the Triton X-100-insoluble fraction of schizont extracts (5). To unambiguously confirm that PSIUB-1 activity can be reliably detected in parasite extracts using the new small synthetic substrates, mature schizonts were extracted with Triton X-100, and the insoluble fraction was incubated with substrate PEP1. Fig. 5 shows that the extracts contained a protease activity that cleaved PEP1 with precisely the same specificity as rPSUB-1. The activity was calcium-dependent, and was poorly inhibited by PMSF, but was pHMB- and DCI-sensitive. The activity was not present in control extracts from uninfected red blood cells (not shown). Remarkably, PEP1 cleavage resulted in only two dominant cleavage products, both of which appeared quite stable to further digestion, suggesting the absence from the parasite extracts of significant additional nonspecific endopeptidase or exopeptidase activity capable of hydrolyzing this substrate or its cleavage products. To ascertain whether the detected activity was accessible to inhibition in intact schizonts by exogenous, membrane-permeable inhibitors, intact schizonts were pretreated with 1 mM pHMB or 2 mM PMSF prior to extraction. Both of these reagents have been previously shown to be permeable to uninfected or malaria parasite-infected red blood cells (29, 30). Pretreatment with pHMB prior to extraction almost completely depleted the activity (Fig. 5H), whereas pretreatment with PMSF had no effect (Fig. 5F). To characterize this parasite activity further, attempts were made to solubilize it. Trials with a number of different detergents (not shown) indicated that the activity could be solubilized from the Triton X-100-insoluble schizont fraction with a buffer containing 5 mM CHAPS and 1 mM NaCl. The PEP1-hydrolyzing activity present in these extracts (Fig. 4A).
6A) could be substantially and specifically decreased by immunodepletion with protein G-Sepharose-immobilized antibodies from two different polyclonal antisera specific for PSUB-1. Western blot analysis of the CHAPS extracts before and after immunodepletion showed that they contained p47, which was mostly removed by the immobilized antibodies (Fig. 6B). We conclude that the parasite-derived PEP1-hydrolyzing activity represents endogenous PSUB-1. It was estimated that the extract from ~6 x 10^8 schizonts contains activity equivalent to 1 μg of rPSUB-1 (data not shown).

Detection of endogenous PSUB-1 activity with substrate PEP1, followed by RP-HPLC-mediated confirmation of cleavage at the correct Asp-Asn bond, provides an indication of authentic PSUB-1-derived activity that is intrinsically more reliable than the use of the fluorescent pepF1-6R substrate, because cleavage of the latter at any position in the peptide backbone can result in a fluorescence increase (10), and spurious hydrolysis by other endogenous proteases could be therefore easily mistakenly ascribed to PSUB-1. However, encouraged by the above indication that the parasite extracts contained no detectable peptidase activities other than PSUB-1, we went on to test whether pepF1-6R could be confidently used to quantify PSUB-1 activity in the extracts. Schizont extracts were supplemented with pepF1-6R, and following a period of incubation at 37°C to allow hydrolysis, the mixture was subjected to RP-HPLC. In addition to residual intact pepF1-6R, only two new products were obtained (not shown), and they exhibited precisely the same retention properties as the products of pepF1-6R cleavage by rPSUB-1 (described elsewhere; also see Ref. 10). We conclude that schizont extracts produced in the manner described contain no peptidase activities capable of cleaving PEP1 or pepF1-6R other than endogenous PSUB-1, and therefore either substrate can be used with confidence to quantify endogenous PSUB-1 activity in parasite extracts.

**Sequence Alignment and Homology Modeling of the PfSUB-1 Catalytic Domain**—Fig. 7 shows a comparison of the PSUB-1 primary sequence with the catalytic domains of four closely related bacterial subtilisins, all of the structures of which have been solved by x-ray crystallography (31–34). The alignment also includes the deduced sequence of a P. yoelii gene very similar to pfsub-1, identified during BLAST searches of preliminary sequence data produced by The Institute for Genomic Research/Naval Medical Research Center. This sequence exhibits 58% overall identity to PSUB-1, including the complete conservation of all 7 cysteine residues within the predicted catalytic domain and very likely represents the P. yoelii orthologue of PSUB-1. It is therefore referred to in the alignment as PySUB-1. To establish the identity of a single unassigned base in the original data and to confirm part of the gene sequence, a DNA fragment encoding Glu^{162}–Ile^{272} of the PySUB-1 sequence was amplified by PCR from P. yoelii genomic DNA and fully sequenced, as described under "Experimental Procedures." This confirmed the nucleotide sequence within this region and showed that residue 228 of the PySUB-1 sequence is an Asn residue, which is of particular interest because this corresponds to Asp^{251} of PSUB-1, the site at which intramolecular processing occurs to form p47 (Fig. 7). Also of note in the deduced PySUB-1 sequence is the conservation of residues equivalent to Val^{216} and Asp^{219} of PSUB-1, adjacent to the p54 processing site, and to Val^{248}, which is in the P4 position relative to the p47 processing site. These observations are all consistent with the above experimental data, suggesting that the P4 Val is rather more critical than the P1 Asp in determining the efficiency of substrate cleavage by PSUB-1. The PySUB-1 sequence shares all the other dominant features of PSUB-1, including the presence of a relatively polar pro-domain sequence (corresponding to Lys^26–Asp^{219} of PSUB-1) and a short C-terminal extension following the predicted catalytic domain. Within the pro-region the malarial sequences are 51% identical, with the most outstanding difference being the presence of an Asn- and Ser-rich insertion in PSUB-1. Interestingly, sequencing of the pfsub-1 gene from a
number of *P. falciparum* clones has shown that this insertion can vary somewhat in length, suggesting that it may not perform an important functional role.

The alignment in Fig. 7 was used to construct a detailed homology-based molecular model of the PfSUB-1 catalytic domain, which is predicted to extend from Phe341 to Asn664 of the PfSUB-1 sequence. This part of the sequence is renumbered in Fig. 7 to allow easy comparison with the accepted numbering system for subtilisin BPN'/H11032 (2). The alignment and modeled structure (contact the corresponding author for the atomic coordinates of the model) predicts a number of noteworthy features. The PfSUB-1 catalytic domain possesses all of the conserved secondary structure elements that make up the core subtilase framework as defined by Siezen and Leunissen (2) and Siezen et al. (21). These include the two prominent surface α-helices (hD, residues Leu104–Ser116, and hE, Phe135–Arg144; subtilisin BPN' numbering used here and henceforth), which in the case of subtilisin BPN' have been shown to form the major site of interaction with the pro-peptide (32, 35). The first of these helices in PfSUB-1 contains two cysteine residues, Cys110 and Cys114, capable of forming disulfide bridges with two spatially adjacent residues, Cys93 and Cys29, respectively, one of which lies in each of two underlying β-strands. A third disulfide bond is predicted between Cys163 and Cys195, mimicking a similarly placed disulfide in the bacterial subtilase aqualysin (36). The predicted existence of three intramolecular disulfide bonds in the PfSUB-1 catalytic domain is consistent with the experimentally demonstrated reduction-sensitive mobility of parasite-derived p54 and p47 on SDS-PAGE (4). This leaves a single free cysteine, Cys156, adjacent to the oxyanion hole residue Asn155 within the active site groove of the structure. It is possible that it is this free cysteine that renders the protease sensitive to inhibition by the sulfhydryl-reactive compound pHMB. Relative to the bacterial subtilisins, the malarial se-

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*Fig. 7. Primary sequence alignment of PfSUB-1 catalytic domain.*

Multiple alignment of the complete PfSUB-1 and deduced PySUB-1 sequences with the catalytic domains of the following four bacterial subtilases: IST3, *Bacillus lentus* subtilisin BL (31); ISCl, a S221C mutant of subtilisin E (32); 1BH6, subtilisin DY, a random mutant of subtilisin Carlsberg (33); and ISUP, *Bacillus amyloliquifaciens* subtilisin BPN' (34). Each subtilisin acronym refers to the respective Protein Data Bank accession number. Residue numbering below each line refers to that of mature subtilisin BPN' (34), whereas that at the right-hand side of lines refers to the complete sequences of the malarial proteases. Dark shading represents predicted or structurally determined α-helix, whereas light shading represents β-strand. Predictions are as output by PHD, with no subsequent manual manipulations (16). The positions of active site residues Asp32, His64, Ser221, and the oxyanion hole residue Asn155 (subtilisin BPN' numbering) are indicated (*). The position of the predicted free cysteine (Cys156 using subtilisin BPN' numbering) in the PfSUB-1 and PySUB-1 sequences is marked (†), as is the extra predicted strand inserted between residues 180–190. Selected specificity-determining residues of the S1 and S4 pocket are marked (!), and | to | indicates disulfide connectivity. The positions at which cleavage occurs to convert the full-length PfSUB-1 protein to p54 and p54 to p47 are marked with arrows.
the side chains of residues 152, 154, 156, and 166. Strikingly, in the S1 pocket and that is probably stabilized by the Cys163 Cys195 disulfide bridge. In subtilisin BPN\(^{\ddagger}\), the corresponding Arg\(^2\) in PfSUB-1 and PySUB-1 may perform an equivalent role. In addition, PfSUB-1 and PySUB-1 probably possess the medium affinity Ca\(^2\) site found in many subtilases; at all positions other than those of the completely conserved catalytic and oxyanion hole residues (Asp\(^{32}\), His\(^{64}\), Asn\(^{155}\), and Ser\(^{221}\)) the identity of the corresponding residues in PfSUB-1 are shown in white. Note that all of these residues except that at position 62 are conserved in the PySUB-1 sequence (Fig. 7).

Discernible are three conspicuous insertions within the catalytic domain. The first of these, lying between Gly\(^{77}\) and Gly\(^{88}\), forms a predicted surface loop rich in conserved Asp and Asn residues, which could act as a calcium ion-binding site (indicated in Fig. 7). If so, this would be in addition to at least two other calcium-binding sites in the molecules because both malarial sequences also possess the conserved Asp\(^{77}\) and the Asn-rich 76–81 loop known to be required to form the high affinity Ca\(^1\) calcium-binding site characteristic of many subtilases in families A, B, and E as defined by Siezen and Leunissen (2). The Ca site requires one other calcium coordination ligand, formed by the side chain of Gln\(^{78}\) in subtilisin BPN\(^{\ddagger}\); the corresponding Arg\(^2\) in PfSUB-1 and PySUB-1 may perform an equivalent role. In addition, PfSUB-1 and PySUB-1 probably possess the medium affinity Ca\(^2\) site found in many subtilases; this requires essential side chain coordination from Asn\(^{106}\), Asn\(^{20}\), and Ser/Thr\(^{74}\) and stabilization by the positively charged side chain of Lys\(^{34}\), all of which are present in both malarial sequences. The second major insertion in the PfSUB-1 catalytic domain lies between residues 162 and 173, in a region that contains several of the specificity-determining residues of the S1 pocket and that is probably stabilized by the Cys163–Cys185 disulfide bridge. In subtilisin BPN\(^{\ddagger}\) the predominantly hydrophobic characteristics of the S1 pocket are provided by the side chains of residues 152, 154, 156, and 166. Strikingly, in both PfSUB-1 and PySUB-1 all of these positions are occupied by serine residues (Figs. 7 and 8), except that at 156, which is replaced by the free Cys\(^{156}\) discussed above. This is likely to result in a relatively polar S1 subsite. In contrast, the S4 pocket of PfSUB-1 is probably a rather hydrophobic environment, because all of the major predicted contributing side chains at positions 126, 128, 135, 104, 102, and 96 (all of which are again conserved between the two malarial sequences) are from either Gly, Leu, or Phe residues (Fig. 8). Phe\(^{128}\) could act as a flexible “flap” above the S4 pocket, as has been suggested for Tyr\(^{104}\) in subtilisin BPN\(^{\ddagger}\) (37). The third insertion unique to the malarial sequences lies between residues 187 and 188, forming a relatively nonpolar loop that is predicted to lie at the surface of the molecule. It is conceivable that this structure may be partly responsible for the tendency of the purified recombinant protease to aggregate.

Discussion

PfSUB-1 is the first malarial serine protease to be expressed in an enzymatically active form. Expression absolutely required the production of a “recom­oned” synthetic gene, pfsub-1\(_{\text{synth}}\). This was originally designed for expression in P. pastoris, but expression in this system was unsuccessful. It is not clear why this is the case, but it may be significant that, to our knowledge, only a single subtilisin-like protease has been expressed in P. pastoris (38), despite the fact that the yeast probably possesses its own endogenous kexin-like protease (39). In the baculovirus system rPfSUB-1 is secreted predominantly in the 54-kDa primary processed form, some of which remains complexed to its cognate pro-peptide. Because subtilase pro-peptides are often potent inhibitors of enzyme activity (e.g. 40–42), it might be expected that such a complex would be enzymatically inactive in trans. If so, the result would be an enzymatically heterogeneous product, with only a proportion of the secreted product (i.e. the fraction of the 54-kDa processed fragment not bound to its cognate pro-peptide) being enzymatically active. The gel filtration data presented here support this, because the most active component of the protein eluted from the column possessed an apparent molecular mass of ~54 kDa, suggesting that it corresponds to free bp54-His\(_{8}\).

Enzymatic characterization of rPfSUB-1 showed that the protease has a slightly alkaline pH optimum and a requirement for unusually high levels of calcium. One interpretation of the latter observation is that PfSUB-1 activity is dependent upon the occupation of a very low affinity calcium-binding site. Many if not most subtilases bind calcium, and this makes a large contribution to the high thermal stability of many extracellular microbial subtilisins (43, 44). Modeling of the PfSUB-1 catalytic domain indicates the presence of the commonly occurring Ca1 and Ca2 sites and in addition a surface loop rich in Asp and Asn residues that are conserved in PySUB-1 and may form a third calcium binding site. Although this loop is predicted to lie some distance from the active site groove of the enzyme, it is possible that its occupancy may be required for optimum enzyme activity. The inclusion of excess calcium during purification might help to stabilize the protease, but this possibility was not explored during this study because of the required use of phosphate buffers in the initial chromatography steps. As in the case of the Ca3 site in subtilisin BPN\(^{\ddagger}\) (45), the site may not be completely selective for calcium, because titration with magnesium or sodium had a small but significant inhibitory effect on PfSUB-1 activity, possibly indicating that these ions are capable of occupying the site without exerting favorable allosteric effects on catalysis. The subcellular environment in which PfSUB-1 needs to exert its physiological role is unknown; it appears to be secreted from the merozoite in a truncated, soluble form at around the point of erythrocyte invasion (4), but it is conceivable that it may perform its function within the dense granules, for example in mediating proteolytic processing of other dense granule components. Nothing is known of the microenvironment within these or any other secretory organelles of the malaria parasite with respect to free calcium levels or pH.

Using several variants of the synthetic peptide substrate PEP1, we have shown that substrate recognition by PfSUB-1 is not prevented by replacement of the P1 Asp with Asn, Glu, or...
Ala, although in the latter two cases small but significant effects on $K_{\text{m}}(\text{app})$ and/or $V_{\text{max}}$ were apparent. Taken together with similar conclusions from the use of *in vitro* translated protein substrates, our results indicate that the P1 residue does not play a primary role in determining the specificity of PISUB-1. The presence of a P1 Leu, in contrast, rendered the substrates virtually uncleavable whether in the form of a peptide or polypeptide and prevented efficient autocatalytic processing of a *psub*-1 synth/D219L mutant expressed in the baculovirus system. Similarly, replacement of the P4 Val in PEP1 with Lys rendered the peptide completely resistant to cleavage, whereas its replacement with Ala resulted in a marked decrease in cleavage efficiency, reducing the $V_{\text{max}}/K_{\text{m}}(\text{app})$ value by ~25-fold. The alignment and three-dimensional model of the PISUB-1 catalytic domain allows us to tentatively reconcile these experimental data with the predicted structure of the PISUB-1 active site cleft. The predominately polar S1 pocket would be expected to readily accommodate an Asn P1 side chain, but the methyl side chain of Ala or the longer side chain of Glu would be less favorable, and the relatively bulky hydrophobic side chain of a Leu residue could not be accommodated. Similarly, the aliphatic side chain of a P4 Val is probably required for efficient hydrophobic interactions with enzyme-derived aromatic and aliphatic side chains in the S4 pocket. The model does not allow any firm predictions of the role of the P2 and P3 residues in substrate recognition, and this issue was not examined experimentally here. However, the alignment of the PFSUB-1 and PySUB-1 sequences (Fig. 7) indicates that the p54 cleavage site in PySUB-1 is on the C-terminal side of the motif LVGAD, suggesting that a P3 Gly may be as readily accommodated as a P3 Ser. More work is required to establish the fine specificity of substrate recognition by PISUB-1, but whereas the bacterial subtilisins as a whole constitute a group of fairly promiscuous degradative enzymes with a broad range of substrate specificities, it is clear that, despite the apparently minor role played by the P1 residue, PISUB-1 displays the relatively high specificity characteristic of eukaryotic processing subtilases. This is probably best illustrated by its capacity to cleave *in vitro* translated PISUB-1 only at the authentic Asp$^{219}$-Asn$^{220}$ site (Fig. 4 and Ref. 5). One significant aspect of the observation that the enzyme cannot cleave a Leu-Asn bond is that it effectively rules out PISUB-1 as a candidate for the parasite serine protease responsible for the well characterized processing of merozoite surface protein-1, an abundant parasite surface protein that is shed during erythrocyte invasion as a result of cleavage at a membrane-proximal Leu-Asn bond (1, 46).

Using both PEP1 and the fluorescent substrate pepF1-6R, we have shown that endogenous PISUB-1 activity can be easily detected as a rather abundant component of extracts of mature *P. falciparum* schizonts. This has important practical implications. It will now be possible to rapidly establish whether compounds isolated from, for example, high throughput screens of combinatorial discovery libraries are equally as effective against authentic PISUB-1 as against the recombinant enzyme. The biological consequences of PISUB-1 inhibition are unknown, so the focus of much future work will be to identify low molecular weight inhibitors of the protease which, if sufficiently selective, will be useful in elucidating the function of the enzyme and may also, if PISUB-1 has potential as a drug target, provide leads for drug development. The presence of a PISUB-1 orthologue in *P. yoelii* raises the possibility of taking advantage of this (and perhaps other) rodent malaria model(s) for *in vitro* testing of lead compounds. This possibility is particularly attractive in view of the apparent complete conservation of all the major specificity-determining residues in PISUB-1 and PySUB-1, suggesting that the two enzymes may have very similar or identical substrate specificities and that inhibitors of PISUB-1 might be equally effective against the *P. yoelii* enzyme. Formal confirmation that PySUB-1 is the functional homologue of PISUB-1 must await genetic complementation studies (47), and this is also a focus of current work. Because PISUB-1 accumulates in dense granules during schizont maturation, effective inhibitors may be required to possess membrane-permeable properties to effectively interfere with its function. Even if, as has been proposed (4), the protease functions predominantly in an extracellular environment during host cell invasion, an ability to readily access endogenous intracellular enzyme may enhance the efficacy of an inhibitory compound by increasing the “therapeutic window” to a period substantially in excess of the brief period of erythrocyte invasion. Here we have shown that the membrane-permeable compound pHMB can very effectively access and inactivate intracellular PISUB-1. The fact that endogenous PISUB-1 is predominantly associated with the Triton X-100-insoluble component of schizont extracts may suggest that the enzyme is associated with other poorly Triton X-100-soluble components of the parasite (e.g. cytoskeletal components) or that it resides within a Triton X-100-resistant subcellular compartment. Little is known of the detergent solubility of the bounding membranes of the various secretory organelles of the malaria parasite, and it may be that dense granules are resistant to Triton X-100. Isolation of these granules by subcellular fractionation (48) should allow the identification of co-localizing proteins, one or more of which may represent the macromolecular substrate of PISUB-1. Alternatively, it is possible that at high local concentrations the parasite protease behaves similarly to the recombinant enzyme and forms aggregates that are resistant to solubilization. It is not clear why rPISUB-1 behaves in this manner; the overall amino acid composition of PISUB-1 does not display any particular bias toward hydrophobic residues, and the three-dimensional model of the catalytic domain does not suggest the presence of a large hydrophobic surface that might be responsible for the observed aggregation, other than the short nonpolar loop between residues 187 and 188 referred to above. Attempts are underway to improve the solubility of rPISUB-1 by strategic mutagenesis of this and other predicted surface residues to facilitate full structural analysis of the protease.

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