Functional Characterization of the Propeptide of Plasmodium falciparum Subtilisin-like Protease-1*

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Erythrocyte invasion by the malaria merozoite is prevented by serine protease inhibitors. Various aspects of the biology of Plasmodium falciparum subtilisin-like protease-1 (PfSUB-1), including the timing of its expression and its apical location in the merozoite, suggest that this enzyme is involved in invasion. Recombinant PfSUB-1 expressed in a baculovirus system is secreted in the p54 form, noncovalently bound to its cognate propeptide, p31. To understand the role of p31 in PfSUB-1 maturation, we examined interactions between p31 and both recombinant and native enzymes. CD analyses revealed that recombinant p31 (rp31) possesses significant secondary structure on its own, comparable with that of folded propeptides of some bacterial subtilisins. Kinetic studies demonstrated that rp31 is a fast binding, high affinity inhibitor of PfSUB-1. Inhibition of two bacterial subtilisins by rp31 was much less effective, with inhibition constants 49–60-fold higher than that for PfSUB-1. Single (at the P4 or P1 position) or double (at P4 and P1 positions) point mutations of residues within the C-terminal region of rp31 had little effect on its inhibitory activity, and truncation of 11 residues from the rp31 C terminus substantially reduced, but did not abolish, inhibition. None of these modifications prevented binding to the PfSUB-1 catalytic domain or rendered the propeptide susceptible to proteolytic digestion by PfSUB-1. These studies provide new insights into the function of the propeptide in PfSUB-1 activation and shed light on the structural requirements for interaction with the catalytic domain.

Parasites of the genus Plasmodium are responsible for the debilitating disease malaria, which affects 300–500 million people each year, mostly in subtropical regions (1). The continued emergence of drug-resistant parasites imposes an urgent need for a new generation of control measures. The life cycle of the malaria parasite in the human is complex, involving intracellular replication in both hepatocytes and erythrocytes, with the pathophysiological manifestations closely allied to intraerythrocytic replication. Plasmodium merozoites actively penetrate erythrocytes via a tightly regulated series of events involving proteolytic processing and release of proteins from the apical organelles and surface of the parasite (2–4). Functional maturation of many invasion-related proteins requires their proteolytic modification and, in some cases, their eventual removal from the parasite surface. These processing events can occur both in the apical organelles and at the parasite surface (4–6), suggesting a role for proteases in different compartments of the parasite. Specific inhibitors of cysteine and serine proteases can block invasion, providing compelling evidence that these classes of parasite proteases play a major role in the invasion process (6, 7). In the case of Plasmodium falciparum, the species responsible for most fatal cases of malaria, two subtilisin-like serine proteases (subtilisins) have been identified, called PfSUB-1 1 and PfSUB-2 (8–10). PfSUB-1 is expressed in the latter stages of schizogony, is located in a subset of dense granules at the apical end of the merozoite, and is secreted during erythrocyte invasion (8), consistent with an involvement in invasion. Sequence comparisons and homology modeling have shown that PfSUB-1 is structurally most closely related to the bacterial subtilisins; unlike most bacterial subtilisins, however, PfSUB-1 has a highly restricted substrate specificity, suggesting a highly specialized, non-degradative physiological role in the parasite (11, 12). It may be a suitable target for drug development. A better understanding of the function, enzymology, and specificity of PfSUB-1, along with the characterization of endogenous inhibitors, may facilitate the development of specific inhibitors as a novel antimalarial therapy.

Subtilases are synthesized as zymogens, which consist minimally of a signal peptide, a propeptide domain, and a catalytic domain. The propeptide functions as an intramolecular chaperone, being essential for correct folding of the catalytic domain and for enzyme maturation (13). Folding is generally rapidly followed by autoproteolytic cleavage at the junction between the propeptide and mature domain. The cleaved propeptide often remains transiently associated with the catalytic domain in a noncovalent complex. In many cases, subtilase propeptides are potent inhibitors of their cognate catalytic domains, with inhibition constants in the nanomolar range (14–18), and so eventful enzyme activation generally requires further proteolytic digestion and release of the propeptide (14, 15). PfSUB-1 maturation during secretory transport in the parasite involves two sequential autocatalytic processing events that convert the precursor first to a p54 form by cleavage of the propeptide and subsequently to a p47 form by further cleavage within the N-terminal segment of p54 (11). Interestingly, when produced in a baculovirus expression system, recombinant PfSUB-1 (rpPfSUB-1) is mostly secreted in the p54 form, a substantial

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of which is noncovalently linked to its cognate protepeptide, p31 (11, 12).

To better understand the activation of PfSUB-1, we have examined the role of the protepeptide and explored the possibility that it can act as a specific inhibitor. After recombinant expression and characterization of the protepeptide, we have investigated its structure, its interactions with both baculovirus-derived and native enzymes, and the specificity and stability of these interactions. Analyses of various mutants of the protepeptide have indicated the regions and residues that determine its interactions with the catalytic domain.

**EXPERIMENTAL PROCEDURES**

**Construction of an Escherichia coli PfSUB-1 Propeptide Expression System**—DNA encoding the propeptide of PfSUB-1 (Lys1–Asp326) of the 690-residue-long primary sequence (8, 11) was amplified by PCR from a synthetic gene (12) using the oligonucleotide primers 5′-AGAGGAGAGTTAGAGCCTTAATCAGCGGACACCAACCTTGTC-3′ (called F1) and 5′-AGAGGAGAGTTAGAGCCTTAATCAGCGGACACCAACCTTGTC-3′ and cloned into pET-30Xa/LIC (Novagen) by ligation-independent cloning according to the manufacturer’s instructions. The recombinant protepeptide was called rp31. A truncated version of rp31 (called rp31A) lacking 11 residues at the C terminus was also generated by PCR using the F1 primer and an rp31A-specific reverse primer, AGAGGAGAGTTAGAGCCTTAATCAGCGGACACCAACCTTGTC-3′. The nucleotide sequences of the cloned products were confirmed on both strands by sequencing on an automated sequencer (Applied Biosystems Model 377).

**Site-directed Mutagenesis**—The desired mutations within the rpSUB-1 protepeptide were introduced by PCR-mediated site-directed mutagenesis using the F1 forward primer in combination with the reverse primers indicated below. Three different rp31 mutants (called rp31-VSAL, rp31-ASAD, and rp31-ASAL) were generated. In the rpSUB-1 propeptide were introduced by PCR-mediated site-directed mutagenesis using the oligonucleotide primers 5′-AGAGGAGAGTTAGAGCCTTAATCAGCGGACACCAACCTTGTC-3′ and cloned into pET-30Xa/LIC (Novagen) by ligation-independent cloning according to the manufacturer’s instructions. The recombinant protepeptide was called rp31. A truncated version of rp31 (called rp31A) lacking 11 residues at the C terminus was also generated by PCR using the F1 primer and an rp31A-specific reverse primer, AGAGGAGAGTTAGAGCCTTAATCAGCGGACACCAACCTTGTC-3′. The nucleotide sequences of the cloned products were confirmed on both strands by sequencing on an automated sequencer (Applied Biosystems Model 377).

**Protein Expression and Purification and Antiserum Production**—rp31 and mutant rp31 sequences were expressed in E. coli BL21-Gold (DE3) pLysS (Stratagene). One liter of LB medium containing 30 μM leupeptin, and 100 μM aprotinin was allowed to stabilize to 37 °C and 0.05% (w/v) Nonidet P-40 was added to a 3 × 3-mm path length cell. The solution was detected by SDS-PAGE (19) and staining with Coomassie Blue R-250. Pooled peak fractions were loaded onto a 1-ml RESOURCE Q column (Amersham Biosciences) pre-equilibrated in 20 mM Tris-HCl (pH 8.2), 12 mM CaCl2, and 10% of the total substrate hydrolysis rate was measured for an additional 5 min. All experiments were performed in triplicate and used concentrations of pepF1-6R well below its Km, under which conditions, Vmax/kg = Vmax (22). In the absence of inhibitors, the rate of hydrolysis over this period was linear, and ~10% of the total substrate was hydrolyzed. Following each assay, the cell was thoroughly cleaned by treatment with chromic acid. P. falciparum schizont extracts containing endogenous PFSUB-1 were prepared by successive solubilizations with Triton X-100 and CHAPS as described previously (11, 12). To determine inhibition of native PFSUB-1 by rp31, the schizont extract was incubated with an unlabeled synthetic peptide substrate called PEP1 (2 mM final concentration) in 50 mM Tris-HCl (pH 8.2), 12 mM CaCl2, and 0.05% (w/v) Nonidet P-40 was added to a 3 × 3-mm path length cell. The solution was detected by SDS-PAGE (19) and staining with Coomassie Blue R-250. Pooled peak fractions were loaded onto a 1-ml RESOURCE Q column (Amersham Biosciences) pre-equilibrated in 20 mM Tris-HCl (pH 8.2), and 150 mM NaCl, and eluted with 2 gel volumes of 150 mM imidazole in the same buffer. Eluted proteins were concentrated by ultrafiltration at 4 °C by using a YM-3 membrane (Amicon, Inc.) and then chromatographed on a Superdex 200 membrane (Amicon, Inc.) and then chromatographed on a Superdex 200 protease purification were as described previously (12). Cleavage of the His6 and S Tags from rp31—The N-terminal fusion peptide containing the His6 and S tags was removed from rp31 using Factor Xa (Roche Applied Science). A solution of purified rp31 was adjusted to 50 mM Tris-HCl (pH 8.2), 100 mM NaCl, and 1 mM CaCl2. Factor Xa was added to obtain a protease/substrate ratio of 1:56 (w/w); and digestion was allowed to proceed at room temperature for 4 h. Protease activity was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. An equal volume of nickel-nitrioltriacetic acid-agarose was added to the digest, and unbond material containing cleaved rp31 (rp31ΔSH) was recovered, supplemented with 10 mM EGTA, and purified by filtration as described above. The rp31ΔSH protein was concentrated by ultrafiltration at 4 °C using a YM-3 membrane (Amicon, Inc.) and stored at −70 °C.

**Determination of Protein Purity and Concentration**—The amino acid composition and concentration of purified rp31 were determined in duplicate amino acid analyses by Dr. Peter Sharratt (Protein and Nucleic Acid Chemistry Facility, Cambridge University, Cambridge, UK). The concentrations of the various purified rp31 mutants were then determined by a comparative reversed-phase high pressure liquid chromatography (RP-HPLC) method. Samples of purified rp31 and its mutant forms were chromatographed on a Vydac C18 RP-HPLC column (4.6 mm × 25 cm) and eluted with a 0–2% (v/v) gradient of acetonitrile in 0.2% TFA for 60 min. Each recombinant protein eluted as a single peak under these conditions. The concentration of each mutant was extrapolated by comparing its peak height upon RP-HPLC with the peak height of a known amount of rp31.

**CD Measurements**—CD spectra of rp31 and rp31ΔSH were recorded from 260 to 195 nm (or from 260 to 210 nm in the presence of urea) at 20 °C in a fused silica cuvette of 1-mm path length using a Jasco J-715 spectropolarimeter. Protein concentrations were ~0.1 mg/ml, and the buffer was 5 mM Tris-HCl (pH 8.2) and 67.5 mM NaCl with or without 5 μM urea (for rp31) or 16.6 mM Tris-HCl (pH 8.2) and 125 mM NaCl (for rp31ΔSH). The spectra are the averages of multiple scans recorded using a scan speed of 200 nm/min and a response time of 0.25 s. Appropriate buffer blanks were subtracted from all spectra. For deconvolution, spectra were subjected to multicomponent secondary structure analysis using the SELCON3 algorithm (20).

**PFSUB-1 Enzyme Activity and Inhibition Assays**—Kinetic experiments assessing the effect of recombinant protepeptide constructs on the activity of baculovirus-derived rPFSUB-1 were as described previously, based on the cleavage of the tetramethylrhodamine-labeled fluorogenic peptide substrate pepF1-6R (12, 21). Briefly, 90 μl of pepF1-6R (2 μM final concentration) in 50 mM Tris-HCl (pH 8.2), 12 mM CaCl2, and 0.05% (w/v) Nonidet P-40 was added to a 3 × 3-mm path length cell. The solution was detected by SDS-PAGE (19) and staining with Coomassie Blue R-250. Pooled peak fractions were loaded onto a 1-ml RESOURCE Q column (Amersham Biosciences) pre-equilibrated in 20 mM Tris-HCl (pH 8.2), and 150 mM NaCl, and eluted with 2 gel volumes of 150 mM imidazole in the same buffer. Eluted proteins were concentrated by ultrafiltration at 4 °C by using a YM-3 membrane (Amicon, Inc.) and then chromatographed on a Superdex 200 membrane (Amicon, Inc.) and then chromatographed on a Superdex 200 protease purification were as described previously (12).

**Selectivity of Inhibition by rp31**—The selectivity of rp31 as a subtilisin inhibitor was assessed by measuring its capacity to inhibit the activity of subtilisin BPN’ (Sigma P-4789) or subtilisin Carlsberg (Sigma P-4756). The assay was performed using the fluorogenic peptide substrate Z-Ala-t-Ala-t-Pro-t-Phe-p-nitroanilide (Suc-AAPF-pNA) by these proteases, essentially as described by Delmar et al. (23), in 50 mM Tris-HCl (pH 8.2), 12 mM CaCl2, and 0.05% (w/v) Nonidet P-40 at room temperature. Kinetic measurements were performed using a Unicam UV1 spectropho-
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RESULTS

Recombinant Expression and Characterization of the PfSUB-1 Propeptide (rp31)—Plasmid pET-30Xa/LIC carrying the p31 sequence (Fig. 1A) was transformed into E. coli strain BL21-Gold (DE3) pLysS, and protein expression was induced with isopropyl-β-D-thiogalactopyranoside as described under “Experimental Procedures.” The recombinant product (rp31) is predicted to comprise Lys^{26–Asp^{219}} of the PfSUB-1 sequence precisely fused to a 46-residue-long N-terminal extension derived from the expression plasmid and containing a His_{6} tag and an S tag, followed by a Factor Xa cleavage site. The expressed protein was mostly soluble (data not shown) and was purified to homogeneity (Figs. 1B, 2A) using a three-step procedure consisting of chelating nickel, gel filtration, and cation-exchange chromatography. The purified product migrated on SDS-polyacrylamide gel as a single species (Fig. 1A). Electrospray mass spectrometry revealed a molecular mass for the pure product of 8,484.4 daltons. The three lanes correspond to three different loadings of purified rp31, quantified by amino acid analysis. C, shown are far-UV CD spectra of rp31SH under nondenaturing conditions (solid line), rp31 under nondenaturing conditions (gray circles), and rp31 in 5 M urea (white circles). The plot shows CD absorption coefficients calculated on a mean residue weight basis (Δm/e) plotted against wavelength (nanometers). The inset shows a comparison of SDSPAGE-fractionated, Coomassie Blue-stained purified rp31 and rp31SH.

Stability of rp31 and rp31 Mutants—The stability of rp31 and its various mutants to digestion by rPfSUB-1 in the presence of the substrate pepF1-6R was assessed by SDS-PAGE and Western blotting. To 90 μl of 2 μM pepF1-6R in 50 mM Tris-Cl (pH 8.2), 12 mM CaCl_{2}, and 0.05% (w/v) Nonidet P-40 were added 10 μl of purified rPfSUB-1 (61.5 nM final concentration) and 1 μl of recombinant propeptide at various concentrations. The mixture was incubated at 37 °C. At intervals, aliquots were removed, and protease activity was terminated by boiling in Laemmli dissociation buffer containing 100 mM dithiothreitol. All samples were then subjected to SDS-PAGE and electroblotted onto Hybond-C membrane (Amersham Biosciences). The membranes were blocked by incubation in 5% (w/v) nonfat milk in PBS and incubated with mouse anti-rp31 antisera, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG antibody. Antigen-antibody interactions were visualized by enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate, Pierce).

Binding of rPfSUB-1 to rp31 and rp31 Mutants—Recombinant propeptide constructs, along with molecular mass marker proteins (Amersham Biosciences) as controls for nonspecific binding of PfSUB-1, were subjected to SDS-PAGE, and the proteins were either stained with Coomassie Blue R-250 or transferred to Hybond-C membrane. The membranes were blocked by incubation in 5% (w/v) nonfat milk in PBS and then incubated for 1 h at room temperature in PBS containing purified rPfSUB-1 (~3 nM final concentration) before washing and probing with a rabbit antisera specific for the catalytic domain of PfSUB-1 (8), followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody. Antigen-antibody interactions were visualized by enhanced chemiluminescence.

CONCLUSIONS

The propeptide of PfSUB-1 is a 312-residue-long N-terminal extension cleaved from the mature protein. The propeptide is cleaved with a Factor Xa cleavage site and consists of an N-terminal His_{6}-tagged region followed by a Factor Xa cleavage site. The propeptide is independently processed from the mature protein and is not required for the stability of the mature protein. The propeptide is processed by rPfSUB-1 in a manner similar to that of the full-length PfSUB-1 protein, and the processing products are similar to those of the full-length PfSUB-1 protein. The propeptide is processed by rPfSUB-1 in a manner similar to that of the full-length PfSUB-1 protein, and the processing products are similar to those of the full-length PfSUB-1 protein. The propeptide is processed by rPfSUB-1 in a manner similar to that of the full-length PfSUB-1 protein, and the processing products are similar to those of the full-length PfSUB-1 protein.
Inhibitory Effect of rp31 on Recombinant and Authentic Pfsub-1—The ability of rp31 to inhibit the proteolytic activity of Pfsub-1 was determined in kinetic assays using the fluorogenic substrate pepF1-6R (2 μM). Preliminary experiments indicated that the addition of a molar excess of rp31 to an ongoing hydrolysis reaction resulted in immediate conversion to a less steep steady-state linear curve, with no evidence of a hyperbolic transition period, suggesting that the propeptide can act as a typical rapid equilibrium competitive inhibitor. Additional experiments showed that this effect was reproducible and concentration-dependent (Fig. 2A). The apparent tight binding inhibition constant (K_{i(app)}) of the interaction was calculated using the method of Nicklin and Barrett (22), which uses the slope of the progress curve immediately following the addition of rp31 (Fig. 2B). Using this method, the experimentally determined K_{i(app)} was 5.3 ± 0.4 nM (Table I). At the substrate concentration used, which is substantially below its K_m, this is unlikely to be significantly different from the K_i (21, 22). Another E. coli

The Triton X-100-insoluble fraction of P. falciparum schizonts was incubated with the substrate PEP1 (2 mM final concentration) for 4 h at 37 °C in the presence or absence of 84 nM purified rp31 before analysis on the digestion mixture by RP-HPLC as described previously (12). Cleavage of PEP1 by endogenous Pfsub-1 resulted in its conversion to two peptide products that eluted at 9 and 9.5 min. Note that prolonged incubation with the schizont extract resulted in complete conversion to the cleavage products (data not shown).
derived recombinant fusion protein possessing the same N-terminal fusion partner as rp31, but containing an irrelevant malarial protein sequence, displayed no detectable inhibitory activity against rPfSUB-1 (data not shown).

To evaluate any effect of the N-terminal His 6 and S tag-containing sequence on the inhibition mediated by rp31, we next investigated the inhibitory properties of rp31ΔSH, which comprises exclusively the PfSUB-1 propeptide sequence (Lys26–Asp219). The determined $K_{\text{app}}$ for inhibition by rp31ΔSH was 12.5 nM (Table I). This value is slightly higher than that found for rp31, but is still in the low nanomolar range and confirms that the inhibition mediated by rp31 is due to the propeptide component of its sequence. The difference between this value and that found for intact rp31 is most likely the result of experimental error in determination of the protein concentration of rp31ΔSH; as shown in Fig. 1C (inset), there was a degree of unavoidable heterogeneity in the rp31ΔSH preparation, presumably caused by limited cleavage at secondary sites during digestion of rp31 with Factor Xa. The presence of minor truncated species in the preparation that do not interact with rPfSUB-1 would have the effect of lowering the specific activity of the preparation. Additionally, any weakly interacting forms might compete with intact rp31ΔSH for binding to the protease, also effectively leading to an underestimate of the inhibition constant. Whatever the case, our data show unambiguously that the PfSUB-1 propeptide is a fast binding, high affinity inhibitor of rPfSUB-1.

We have previously shown that the Triton X-100-insoluble fraction of P. falciparum schizont extracts contains endogenous PfSUB-1 activity (11, 12). The addition of the unlabeled peptide substrate PEP1 to these extracts results in its conversion to two dominant cleavage products diagnostic of cleavage by PfSUB-1. Treatment of the schizont extract with purified rp31 was found to completely deplete the activity (Fig. 2C). This result shows that the activity of authentic, parasite-derived PfSUB-1 can be potently inhibited by the propeptide. This result is of particular interest because, whereas baculovirus-derived rPfSUB-1 is secreted in the p54 form, endogenous PfSUB-1 in schizont extracts is predominantly in the form of the terminal p47 processing product (11, 12). Our results therefore indicate that rp31 is able to bind to and inhibit both p54 and p47 forms of PfSUB-1, showing, in turn, that conversion of p54 to p47 does not per se abolish the ability of the protease to interact with its propeptide.

Selectivity of Inhibition by rp31—The PfSUB-1 catalytic domain is phylogenetically most closely related to the families of bacterial subtilisins and bacterial pyrolysin subtilases (8, 12, 27). Members of these families sharing the highest amino acid homology with PfSUB-1 include subtilisin BPN’ and subtilisin Carlsberg (32 and 31% identities, respectively, within the catalytic domains) (12). To determine whether the inhibitory activity of rp31 is selective for its cognate enzyme, we investigated its effect on the activity of these two bacterial subtilisins in kinetic assays by monitoring the effects of rp31 on hydrolysis of Suc-AAPF-pNA as described under “Experimental Procedures.” Inhibition of both enzymes by rp31 was substantially poorer than that seen with rPfSUB-1, with calculated $K_{\text{app}}$ values of 0.33 ± 0.02 μM for subtilisin BPN’ and 0.36 ± 0.05 μM for subtilisin Carlsberg. The experimentally determined $K_m$ values for cleavage of Suc-AAPF-pNA by these proteases under the conditions used were 242 ± 8 μM for subtilisin BPN’ and 434 ± 51 μM for subtilisin Carlsberg (data not shown), so the above $K_{\text{app}}$ values convert to $K_m$ values of 0.06 μM for subtilisin BPN’ and 0.07 μM for subtilisin Carlsberg (22). Relative to inhibition of rPfSUB-1 ($K_m$ = 5.3 μM), these values correspond to selectivity indices of ~49 and 60, respectively. Our data suggest that rp31 is a relatively selective inhibitor of its cognate enzyme.

Effect of Point Mutations and C-terminal Truncation on the Inhibitory Properties of rp31—The S1 and S4 binding pockets are thought to dominate substrate preference in subtilisins (27). Examination of the amino acid sequences flanking the cleavage sites used during autocatalytic processing of PfSUB-1 has revealed two conserved residues at the P1 (Asp) and P4 (Val) positions relative to the scissile bonds (11). Subsequent studies have shown that, although the P1 Asp is not crucial for substrate cleavage, it is the preferred residue at this position for efficient substrate cleavage in trans by PfSUB-1, and its replacement with Leu completely prevents cleavage (12). Similarly, the P4 Val plays an important role in substrate recognition (12). On the basis that these residues might also be important for the inhibition mediated by rp31, we generated, by mutagenesis, point mutations in appropriate positions of the recombinant propeptide. Three mutant forms of rp31 were investigated: a mutant with replacement of P4 Val with Ala (rp31-ASAD), a mutant with replacement of P1 Asp with Ala (rp31-ASAL), and a double mutant with replacement of both P1 Asp and P4 Val with Ala (rp31-ASAL) (Fig. 1; see Fig. 4A, third through fifth lanes).

Inhibition assays with the mutant proteins showed that all remained potent inhibitors of rPfSUB-1, with calculated $K_{\text{app}}$ values of 5.1 ± 0.3, 2.6 ± 1.3, and 11.9 ± 3.6 nM for rp31-ASAD, rp31-ASAL, and rp31-ASAL, respectively (Table I). These results were unexpected, indicating that the modified residues play little if any role in the potent inhibition mediated by the propeptide. To further explore the mechanism of inhibition by rp31, we therefore constructed, expressed, and purified to homogeneity another construct identical to rp31 except for the truncation of 11 residues (Leu209–Asp219) from its C terminus (Fig. 1; see Fig. 4A, second lane). Surprisingly, this construct (called rp31Δ11) also inhibited the activity of rPfSUB-1, although with a considerably reduced efficiency compared with rp31 or the point mutants described above, with a calculated $K_{\text{app}}$ of 76.6 ± 15.9 nM (Table I). This indicates a requirement for interactions between the C-terminal “tail” of the propeptide and PfSUB-1 for optimal inhibition, but also shows that other interactions involving residues upstream of this domain play an important role in binding of the propeptide to the catalytic domain.

Stability of rp31 and rp31 Mutants—The propeptides of certain subtilisins are readily and rapidly digested by their cognate enzymes. One practical consequence of this is that it can lead to potential inherent errors when attempting to experimentally define inhibition constants due to a time-dependent decrease in the concentration of the propeptide (e.g. Ref. 28). Although rp31 and its various mutants did not exhibit any discernible time-dependent decrease in their inhibitory capacity during the inhibition assays described above, we felt it important to assess the capacity of the propeptide to act as a
substrate for rPISUB-1 and to verify that the concentration of each polypeptide was stable during the assay. To assess this, purified rp31 or the various mutant propeptides were incubated at 37 °C in the presence of the substrate pepF1-6R and rPISUB-1 under conditions identical to those used in the assays performed to measure their inhibitory activity. Two concentrations of each propeptide were tested, corresponding to the lowest and highest concentrations used in the inhibition assays. Fig. 3 shows that, even at the lowest propeptide concentration used, the concentration of each propeptide remained stable over time, and no digestion products could be detected upon SDS-PAGE analysis. Similar results were obtained using the highest concentration of each propeptide used in the inhibition assays (data not shown). These data clearly demonstrate that rp31 is completely resistant to digestion by PISUB-1 over the time course of the assay. Moreover, the truncation and the various mutations made to the propeptide did not reduce this resistance to digestion.

Binding of rp31 and rp31 Mutants to Baculovirus-derived PISUB-1—The above inhibition data clearly imply tight binding of rp31 and its various derivatives to rPISUB-1. To directly visualize this, we used a modification of a binding assay described by Kessler and Safrin (29) and Markaryan et al. (30). The various recombinant propeptides were subjected to SDS-PAGE and electroblotted onto a membrane. The membrane was subsequently incubated with PBS containing purified rPISUB-1 and then probed with a rabbit antisera specific for the PISUB-1 catalytic domain. A single band was observed in each case (Fig. 4B) and the bands corresponded exactly to the position of the corresponding propeptide construct (Fig. 4A). No signal was detected on control blots, which were not probed with rPISUB-1 (Fig. 4C), demonstrating that the signals observed in Fig. 4B were not due to nonspecific recognition of the propeptides by the rabbit antibodies or the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies used. The specificity of PISUB-1 binding was demonstrated using a set of standard molecular mass marker proteins, all loaded at a higher concentration than the propeptide constructs. Two species in the marker lanes, corresponding to phosphorylase b (94 kDa, 640 ng), bovine serum albumin (67 kDa, 830 ng), ovalbumin (43 kDa, 1470 ng), carbonic anhydrase (30 kDa, 830 ng), and soybean trypsin inhibitor (20 kDa, 800 ng), were used as controls for nonspecific binding, their molecular masses, and the amounts loaded onto each lane of the gel were phosphorylase b (94 kDa, 640 ng), bovine serum albumin (67 kDa, 830 ng), ovalbumin (43 kDa, 1470 ng), carbonic anhydrase (30 kDa, 830 ng), and soybean trypsin inhibitor (20 kDa, 800 ng).

DISCUSSION

In several protease families, propeptides function as intramolecular chaperones that are essential for correct folding of the catalytic domain during secretory transport. Propeptides are also often potent inhibitors of their associated catalytic domains and are thought to act as regulators of protease activity during secretory transport, preventing potentially damag-
ing premature activation (13, 29–32). Initial studies on secretion of rPfSUB-1 from insect cells demonstrated that at least a proportion of the secreted protease remains noncovalently linked with its propeptide, p31 (11). These early observations raised the question of the role of p31 in the activation and function of PFSUB-1 and prompted us to examine its structure and activity in detail.

CD analysis of the recombinant propeptide both with (rp31) and without (rp31ASH) an N-terminal fusion peptide derived from the expression plasmid demonstrated that it possesses secondary structure in the absence of its cognate catalytic domain. Previous studies by others have indicated that subtilase propeptides broadly divide into two groups according to their folding capacity. Propeptides of bacterial subtilisin BPN’ and subtilisin Carlsberg are unfolded when expressed on their own (17, 33, 34), whereas propeptides of alycasulins I and of some of the subtilisin-like protease convertases (SPCs) adopt significant secondary structure (18, 35, 36). Thus, in the case of alycasulins I, the isolated propeptide was estimated to be composed of 21% α-helix and 24% β-sheet (35). The propeptides of several SPCs have been shown to possess a similar degree of secondary structure, with, for example, the propeptide of mouse SPC3 being 22% α-helical and 30% β-sheet (18). The observed α-β content of rp31 is in good agreement with these values. It has been suggested that propeptides with significant secondary structure are more stable and more efficient in both folding and inhibiting the catalytic domain than those that lack structure. In the case of subtilisin BPN’, Kojima et al. (33) demonstrated, through a series of amino acid replacements, that an increase in secondary structure in the propeptide could be correlated with a slower rate of decrease in the inhibitory activity and a greater resistance to proteolytic digestion in the presence of the cognate catalytic domain, both leading to a lower $K_i$ than that for the wild-type propeptide. Likewise, the alycasulins I propeptide is more efficient in the folding and inhibition of subtilisin BPN’ compared with the subtilisin BPN’ propeptide itself, which lacks structure (35). Unstructured propeptides are thought to adopt structure only upon interaction with their cognate catalytic domains (34, 37), so these observations are consistent with a model in which possession of some pre-existing structure accelerates productive interaction with their cognate catalytic domains (38, 39). A recent NMR structure of the 83-residue-long PC1 propeptide showed that the isolated molecule adopts a fold similar to that of the enzyme-bound bacterial propeptide, despite the absence of any sequence homology to the bacterial protein (36). Homology modeling of the PFSUB-1 catalytic domain has revealed an overall structure similar to that of subtilisin BPN’, including the presence of the two surface α-helices (12), so the manner in which PFSUB-1 interacts with its propeptide is probably broadly similar to that of the bacterial subtilisins. Our finding that point mutations within the four residues at the C terminus of rp31 had little effect on its inhibitory capacity and that even removal of 11 residues from the C terminus did not abolish inhibition (although there was an −14-fold increase in $K_{i(app)}$) was therefore unexpected. Our data clearly demonstrate that, in the case of the PFSUB-1 propeptide, interactions within the active-site cleft may be important for optimal inhibition, but are not essential for complex formation. Since inhibition presumably involves steric blockage of the PFSUB-1-active-site groove, it is not clear how this is mediated by the truncated propeptide; however, it is important to note that p31 is substantially larger than any of the subtilase propeptides whose three-dimensional structures have been solved, and it is possible that, in the complex formed between PFSUB-1 and rp31A11, structures formed by propeptide residues distant in the primary sequence from its C terminus can occlude access to the active site.

Some subtilisin propeptides are efficiently degraded by their cognate enzymes (e.g. Refs. 17 and 27). This can cause practical difficulties in kinetic measurements, during which the propeptide concentration needs to remain stable. It may also be important for eventual autocatalytic removal of the propeptide from the protease catalytic domain during activation. Our finding that rp31 was not degraded over the course of the inhibition assays validates the accuracy of the $K_{i(app)}$ values calculated for all of the propeptide constructs. It is also consistent with previous indications that PFSUB-1 has a fairly stringent substrate specificity. Studies using synthetic peptide substrates have indicated that the favored minimal recognition sequence for PFSUB-1 is Val-X-X-Asp ↓, where X is any amino acid residue (12). No such motif occurs within p31 (besides that at the extreme C terminus). This apparent specificity of PFSUB-1 raises the question of how activation of the protease ultimately takes place during trafficking through the parasite secretory transport system since it presumably requires removal of the propeptide. In many cases, including that of the SPC furin, subtilase propeptide removal involves its proteolytic cleavage, mediated by the cognate protease in cis or in trans (40, 41). Our data suggest that this is unlikely to be so in the case of PFSUB-1. We have previously shown that, in the parasite, p54 is bound to its propeptide (11) and that conversion of p54 to p47 occurs only in a post-endoplasmic reticulum compartment (8). Our demonstration here that the propeptide can bind to and inhibit parasite-derived p47 implies that, whatever the function of the conversion of p54 to p47, this processing step is insufficient per se to remove the bound propeptide. Propeptide release may be mediated by a simple environmental trigger (such as a change in pH or calcium concentration) associated with transport beyond the parasite endoplasmic reticulum.
The identity of the physiological substrate(s) of PfSUB-1 is unknown. Given the apparent high substrate specificity of the enzyme, it is unlikely to be involved in degradative processes; a role in specific post-translational protein modifications is more likely, which could take place either within the secretory organelles in which PfSUB-1 accumulates or following protease release during erythrocyte invasion. We currently favor the former possibility since we have found that the addition of high concentrations of rp31 to *P. falciparum* cultures has no effect on the efficiency of erythrocyte invasion, and extensive experiments have indicated that rPSUB-1 has no detectable capacity to modify surface proteins of the intact merozoite or the host red blood cell under nondenaturing conditions. In light of the findings described here, rp31 could be used to regulate the activity of PfSUB-1 in a specific and informative manner; it may be possible, for example, to subtly and specifically down-regulate levels of functional PfSUB-1 in the parasite by constitutive overexpression of the propeptide from a suitable transgene. The demonstration that rp31 inhibits both authentic and baculovirus-derived PSUB-1 validates the use of the recombinant protease as a tool for high throughput screening for low molecular mass inhibitors (21). Defining the important inhibitory sequences in rp31 could aid in developing potent inhibitors with potential as antimarial compounds. Indeed, endogenous inhibitors are generally a good starting point for further development of smaller but highly selective inhibitors (43–47).

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Functional Characterization of the Propeptide of *Plasmodium falciparum* Subtilisin-like Protease-1

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