Plasmepsin II, an Acidic Hemoglobinase from the *Plasmodium falciparum* Food Vacuole, Is Active at Neutral pH on the Host Erythrocyte Membrane Skeleton*

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Plasmepsin II, an aspartic protease from the human intraerythrocytic parasite *Plasmodium falciparum*, is involved in degradation of the host cell hemoglobin within the acidic food vacuole of the parasite. Previous characterization of enzymatic activities from *Plasmodium* soluble extracts, responsible for *in vitro* hydrolysis of erythrocyte spectrin, had shown that the hydrolysis process occurred at pH 5.0 and involved aspartic protease(s) cleaving mainly within the SH3 motif of the spectrin α-subunit. Therefore, we used a recombinant construct of the erythroid SH3 motif as substrate to investigate the involvement of plasmepsins in spectrin hydrolysis. Using specific anti-plasmepsin II antibodies in Western blotting experiments, plasmepsin II was detected in chromatographic fractions enriched in the parasite SH3 hydrolyase activity. Involvement of plasmepsin II in hydrolysis was demonstrated by mass spectrometry identification of cleavage sites in the SH3 motif, upon hydrolysis by *Plasmodium* extract enzymatic activity, and by recombinant plasmepsin II. Furthermore, recombinant plasmepsin II digested native spectrin at pH 6.8, either purified or situated in erythrocyte ghosts. Additional degradation of actin and protein 4.1 from ghosts was observed. Specific antibodies were used in confocal imaging of schizont-infected erythrocytes to localize plasmepsin II in mature stages of the parasite development cycle; antibodies clearly labeled the periphery of the parasites. Taken together, these results strongly suggest that, in addition to hemoglobin degradation, plasmepsin II might be involved in cytoskeleton cleavage of infected erythrocytes.

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Malaria is a tropical disease caused by the protozoan parasite *Plasmodium*. In 1996, the World Health Organization estimated the annual mortality at 1.7–2.5 million and persons living in risk areas at ~2 billion. Resistance of *Plasmodium* to antimalarial drugs and vector resistance to insecticides are increasing problems in fighting the parasite. New approaches to chemotherapy based on blocking essential metabolic pathways of the parasite are necessary. Toward this end, the parasite proteases that are involved in crucial steps of *Plasmodium* development appear to be good targets. Several have been described that are involved in hemoglobin hydrolysis (the so-called hemoglobinases), antigen maturation, merozoite release, or invasion of new red blood cells by merozoites (1).

Among the *Plasmodium* proteases, the hemoglobinases have been extensively studied (2). In the food vacuole (pH 5–5.4), the 37-kDa aspartic proteases plasmepsins I and plasmepsin II initiate cleavage of the native hemoglobin tetramers, whereas the 28-kDa cysteine protease falcipain appears to digest the denatured or fragmented substrate (3). These three hemoglobinases have been cloned and sequenced (4–6). A 43-kDa recombinant precursor of plasmepsin II lacking the first 76 residues of full-length proplasmepsin II has been expressed in *Escherichia coli*, and its acidification results in the production of a 38-kDa protein by autocatalytic cleavage (7). The 38-kDa enzyme showed kinetic properties similar to those of native plasmepsin II (8). Thus far, no clear difference in structure or function has emerged between plasmepsins I and II, and the reason why the parasite synthesizes both enzymes remains obscure. Unexpectedly, our work on the involvement of proteases in the invasion process yields new insights into the substrate selectivity of plasmepsin II compared with plasmepsin I.

Many years ago, the depletion of spectrin and protein 4.1, two major proteins of the erythrocyte membrane cytoskeleton, was described for infected red blood cells during the schizogonic stage of *Plasmodium berghei* (9). A *Plasmodium lophurae* 37-kDa cathepsin D was reported to digest spectrin and bands 2.1, 2.6, and 3 from the membrane and human red blood cells at both pH 3.5 and 7.4 (10). Also, a 37-kDa acidic proteinase, able to cleave spectrin and protein 4.1 from human erythrocytes at both pH 5.0 and 7.2, was purified from soluble extracts of *P. falciparum* and *P. berghei* (11). In 1996, another *P. falciparum* proteolytic activity, clearly inhibited by 10–20 μM pepstatin A and involving protease(s) in the 35–40-kDa range, was described to act at acidic pH on human spectrin (12). This “spectrinase” activity cleaved the α-spectrin at a site located within the SH3 motif of the molecule.

Owing to the fact that plasmepsins I and II are pepstatin A-inhibitable acidic proteinases with a molecular mass of 37 kDa, we investigated the presence of these hemoglobinases in *P. falciparum* chromatographic fractions enriched for the SH3 hydrolase activity. Plasmepsin II was found in the most active...
fractions. Mass spectrometry identification of cleavage sites in the SH3 motif upon digestion by recombinant plaspepsin II or by spectrinase activity unambiguously established involvement of plaspepsin II in in vitro hydrolysis of the SH3 motif. The recombinant enzyme proved to be able to digest native spectrin at pH 6.8, either purified or in erythrocyte ghosts. Confocal microscopy analysis of infected erythrocytes with anti-plaspepsin II antibodies showed that the enzyme can be visualized outside the parasite in mature stages of Plasmodium. These results strengthen the idea of plaspepsin II being devoted to function(s) other than hemoglobin degradation in infected red blood cells and, more generally, address the question of the redistribution of some specific proteases during the intraerythrocytic development of P. falciparum.

**EXPERIMENTAL PROCEDURES**

**Tools and Chemicals—**Saponin and N'-benzoylcarbonyl-Phe-Arg 7-amido-4-methylcoumarin fluorogenic substrate were purchased from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). Fluorescein isothiocyanate-labeled anti-rabbit Ig antibodies and tetramethylrhodamine-biotinase B isothiocyanate-labeled anti-mouse Ig antibodies were purchased from Nordic Immunological Laboratories. Construction of the recombinant GST-SH3 peptide and linkage to Sepharose beads have been previously described (12).

*P. falciparum in Vitro Culture and Synchronization—* The FcB1 strain from Colombia was used throughout the experiments. Culture was performed according to Trager and Jensen (13). Basic culture medium contained RPMI 1640 medium (Life Technologies, Inc.), 25 mM Hepes, 27.5 mM NaHCO3, and 11 mM glucose (pH 7.4) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 7% (v/v) heat-treated human plasma. Red blood cells from the same donor were added to a hematocrit of 2–3%, and the culture was maintained under an atmosphere of 3% CO2, 6% O2, and 91% N2 at 37 °C, with daily heat-treated human plasma. Penicillin (100 IU/ml), streptomycin (100 μg/ml), and 7% (v/v) heat-treated human plasma were added to a hematocrit of 2–3%, and the culture was maintained under an atmosphere of 3% CO2, 6% O2, and 91% N2 at 37 °C, with daily medium changes. Synchronization of cultures was achieved by Plasma- gell treatment (14), followed, 4–5 h later, by 5% (v/v) sorbitol treatment (15). Under our culture conditions, the in vitro life cycle of the FcB1 strain was 48 h.

**Enzymatic Extracts and Enrichment of P. falciparum Spectrinase Activity—**100,000 × g extracts from the schizont stage of *P. falciparum* were prepared as described by Le Bonniec et al. (12). For enrichment of acidic spectrinase activity, gel filtration of 100,000 × g extracts on Superose 12 and chromatography on alkyl-Sepharose were performed using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). The gel filtration procedure has been described (12). For spectrin hydrolysis through hydrophobic interactions, pooled active fractions from Superose 12 were applied to an alkyl-Sepharose HR 5/5 column equilibrated in 100 mM phosphate buffer (pH 7.2) containing 2 mM ammonium sulfate. Elution was performed with a gradient of ammonium sulfate from 2 to 0 in 100 mM phosphate buffer at a flow rate of 0.5 ml/min. Usually, 1-ml fractions were collected and tested for their activity with GST-SH3 peptide and linkage to Sepharose beads have been previously described (12).

**Mass Spectrometry Analysis—** After digestion of the SH3 motif with either recombinant plaspepsin II or the spectrinase-enriched fraction, the released peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). After digestion, the samples were acidified by performing a dilution (1:2 to 1:10) in a 0.1% aqueous trifluoroacetic acid solution. The MALDI matrix used was 2,5-dihydroxybenzoic acid (Aldrich). The acidified samples were then mixed 1:1 (v/v) with a saturated solution of 2,5-dihydroxybenzoic acid in 0.1% aqueous trifluoroacetic acid and analyzed. After determination of their molecular masses, cleavage sites in the SH3 motif were identified.

MALDI-TOF-MS measurements were performed on a Voyager Elite mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA) in positive ion reflectron mode using a delayed extraction. For laser desorption, a nitrogen laser beam (λ = 337 nm) was used. Typically, 150–256 shots were averaged for each acquired spectrum. External calibration was performed using a mixture of neurotensin, ACTH (18–39), and ACTH (7–38) with m/z ratios corresponding to monoisotopic [M + H]+ of 1672.92 and 2465.20 and average [M + H]+ of 3660.19, respectively. Only the average m/z ratios of the protonated peptides are given under “Results.” The difference between the calculated average mass and the experimental mass determination was <0.5 atomic mass unit.

**SDS-PAGE and Western Blot Analysis—** Electrophoresis was performed on 10 and 15% acrylamide gels according to Laemmli (19). Gels were stained with Coomassie Brilliant Blue R-250. Proteins were electrotransferred onto nitrocellulose membranes according to Towbin et al. (20). Rabbit anti-plaspepsin I and anti-plaspepsin II antibodies (21) were used at a 1:250 dilution.

**Confocal Microscopy Analysis—** Smears of asynchronous cultures of the FcB1 strain were fixed for 2 min at ~20 °C in acetone/methanol (7:3, v/v). Double labeling of cells with anti-plaspepsin II polyclonal antibodies (serum 737 (21); 1:200 in PBS and 3% nonfat milk (pH 7.5)) and anti-MSF1 (merozoite surface protein 1) monoclonal antibodies (ascites 22-2 (22); 1:50 in PBS and 3% nonfat milk (pH 7.5)) was performed by incubations (2 h, room temperature) with the respective antibodies. After washing in PBS and 3% nonfat milk (3 × 10 min), slides were incubated with mixed tetramethylrhodamine B isothiocyanate-labeled anti-mouse Ig antibodies (1:50) and fluorescein isothiocyanate-labeled anti-rabbit Ig antibodies (1:100) in PBS and 3% nonfat milk for 2 h at room temperature. Control slides for labeling of cells with secondary antibodies and nonimmune rabbit serum were processed in parallel. Slides were washed in PBS (3 × 10 min) and mounted in FluoroGuardTM antifade reagent (Bio-Rad) under a coverslip. Observations were made using a confocal laser scanning microscope (MRC 1024, Bio-Rad).

**RESULTS**

**Plaspepsin II, an Acidic Endoprotease from the Food Vacuole of Plasmodium, Co-purifies with the Spectrinase Activity—** Owing to the acidic pH activity of the parasite SH3 hydrolase, we looked for the presence of the three hemoglobinases plaspepsin I, plaspepsin II, and plaspepsin in the fractions recovered by gel filtration of the 100,000 × g extract from the schizont stage of the FcB1 strain of *P. falciparum*. The spectrinase activity was localized in the eluted fractions via hydrolysis of the recombinant GST-SH3 peptide at pH 5.0 and 37 °C and analyzed by SDS-PAGE and Coomassie Blue staining of the gel. In controls for each fraction omitting GST-SH3, the amount of protein in the 30–50-kDa range was too low to be

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1. The abbreviations used are: GST, glutathione S-transferase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MALDI- TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ACTH, adrenocorticotropic hormone; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
detected (data not shown). Plasmepsins I and II were visualized by Western blotting of the fractions using specific anti-plasmepsin I antibodies (serum 574) and anti-plasmepsin II antibodies (serum 737). The presence of falcipain was checked by cleavage of the fluorogenic dipetidyl substrate 4-nitrobenzoyloxycarbonyl-Phe-Arg 7-amido-4-methylcoumarin (23). Distribution of the spectrinase activity (Fig. 1A) showed that fractions 8 and 9 contained the highest SH3-hydrolyzing activity, and plasmepsin II was clearly visualized in these fractions (Fig. 1C). In contrast, plasmepsin I, although also a 37-kDa protein, was visualized in fractions 4–9, peaking in fraction 4 (Fig. 1B), which corresponds to eluted proteins with ~100-kDa molecular masses. As chromatography was performed under nondenaturing conditions, early elution of plasmepsin I might be due to protein-protein interactions. Low SH3-hydrolyzing activity was detected in plasmepsin I-containing fractions compared with plasmepsin II-containing fractions. With respect to falcipain, degradation of the Phe-Arg 7-amido-4-methylcoumarin substrate was observed with fractions 9 and 10 (Fig. 1D) and was minimal in fraction 8, which had high proteolytic activity against the SH3 motif. Also, falcipain is a cysteine protease, hence is not inhibited by pepstatin A, in contrast to what was observed with the spectrinase activity.

To test the hypothesis that the enzyme(s) responsible for SH3 hydrolysis might be different from plasmepsin I, various biochemical procedures were tried to separate plasmepsin II from the SH3 hydrolyase activity. 100,000 × g schizont extract from schizonts of the FcB1 strain. Elution fractions 4–12 (approximately corresponding to the elution of proteins in the range of 100 to 20 kDa) were split into four aliquots. One aliquot was tested for its SH3-hydrolyzing capacity. The recombinant GST-SH3 peptide was incubated with the fraction for 2 h at pH 5.0 and 37 °C. SH3 hydrolysis was analyzed by SDS-PAGE and Coomassie Blue staining of the gel (A). The recombinant GST-SH3 peptide was also incubated at pH 5.0 in the elution buffer alone (control (co)). The presence of plasmepsins I and II was checked by Western blot analysis of two other aliquots from each fraction using anti-plasmepsin I antibodies (1:2500; B) and anti-plasmepsin II antibodies (1:2500; C). The remaining aliquot was used to test the presence of falcipain. Enzymatic activity was checked by cleavage of the fluorogenic dipetidyl substrate N\(^-\)benzoyloxycarbonyl-Phe-Arg 7-amido-4-methylcoumarin, a specific substrate for falcipain. Emitted fluorescent light was collected at 440 nm upon excitation at 380 nm (arbitrary units). Molecular masses (in kilodaltons) are on the right.

**Fig. 1. Identification of the hemoglobinases in Superose 12 fractions of a 100,000 × g extract from schizonts of the FcB1 strain.** Elution fractions 4–12 (approximately corresponding to the elution of proteins in the range of 100 to 20 kDa) were split into four aliquots. One aliquot was tested for its SH3-hydrolyzing capacity. The recombinant GST-SH3 peptide was incubated with the fraction for 2 h at pH 5.0 and 37 °C. SH3 hydrolysis was analyzed by SDS-PAGE and Coomassie Blue staining of the gel (A). The recombinant GST-SH3 peptide was also incubated at pH 5.0 in the elution buffer alone (control (co)). The presence of plasmepsins I and II was checked by Western blot analysis of two other aliquots from each fraction using anti-plasmepsin I antibodies (1:2500; B) and anti-plasmepsin II antibodies (1:2500; C). The remaining aliquot was used to test the presence of falcipain. Enzymatic activity was checked by cleavage of the fluorogenic dipetidyl substrate N\(^-\)benzoyloxycarbonyl-Phe-Arg 7-amido-4-methylcoumarin, a specific substrate for falcipain (D). Emitted fluorescent light was collected at 440 nm upon excitation at 380 nm (arbitrary units). Molecular masses (in kilodaltons) are on the right.

**Fig. 2. Kinetic analysis of GST-SH3 hydrolysis by recombinant plasmepsin II.** Approximately 10 μl of pelleted GST-SH3 substrate linked to Sepharose beads was incubated with 0.5 μg of recombinant plasmepsin II in 25 mM Tris citrate (pH 5.0) (control (co)). After a 2-min (lane 1), 5-min (lane 2), 15-min (lane 3), 30-min (lane 4), 1-h (lane 5), or 20-h (lane 6) incubation, aliquots were taken, boiled in SDS reducing buffer, and then analyzed by SDS-PAGE on a 15% acrylamide gel. The gel was stained with Coomassie Blue. Molecular masses (in kilodaltons) are on the right.

**Fig. 3. Location of Cleavage Sites in the SH3 Motif by Mass Spectrometry Analysis of the Hydrolysis Products.** To unambiguously clarify the involvement of plasmepsin II in the SH3 hydrolysis, the identification of SH3 cleavage sites upon spectrinase action and upon recombinant plasmepsin II action was undertaken by MALDI-TOF-MS analysis of the released fragments. The recombinant GST-SH3 peptide linked to Sepharose beads was incubated at pH 5.0 and 37 °C with recombinant plasmepsin II or with a spectrinase-enriched fraction recovered by gel filtration of a 100,000 × g schizont extract. Hydrolysis products were analyzed by SDS-PAGE on a Tricine gel, allowing the visualization of peptides in the 1–5-kDa range (data not shown). Sepharose beads were pelleted by centrifugation, and the supernatant was analyzed by MALDI-TOF-MS. The identified cleavage sites along the amino acid sequence of the SH3 motif are reported in Fig. 3.

Six sites were identified due to cleavage by the spectrinase-enriched fraction (Fig. 3, sequence 2). They are distributed within three “regions” of cleavage, showing one site (F ↓Q), two sites (Y ↓V, R ↓R) and three contiguous sites (L ↓L, L ↓S, S ↓S) of cleavage, respectively. Location of the cleavage regions along the amino acid sequence corresponds to the expected generation of the 34-, 32-, and 30-kDa proteolytic fragments.

Five sites were identified due to cleavage by recombinant plasmepsin II (Fig. 3, sequence 1). Out of the five, four corresponded to the F ↓Q, Y ↓V, L ↓L, and L ↓S sites identified upon digestion of the SH3 motif by the spectrinase fraction. The fifth site (F ↓I) was located outside the SH3 amino acid sequence, in the recombinant plasmid sequence.

Although mass quantitation of the hydrolysis fragments was difficult by MALDI-TOF-MS, the intensity of some peaks was highly reproducible from one experiment to the other, suggesting that the corresponding sites were highly susceptible to cleavage. From this, the Y ↓V and S ↓S sites appear as major sites of cleavage with the spectrinase fraction, and Y ↓V and L ↓L are major sites with plasmepsin II. Minor sites with the spectrinase fraction and plasmepsin II are R ↓R, L ↓L, and L ↓S and F ↓I and L ↓S, respectively. A very minor site at F ↓Q was revealed for both enzymatic activities.

Two main conclusions must be drawn from these results. First, plasmepsin II is unambiguously and predominately involved in the in vitro hydrolysis of the SH3 motif observed with the parasite spectrinase fraction; and second, enzyme(s) other than plasmepsin II are involved in this hydrolysis, cleaving at the R ↓R and S ↓S sites. To detail the sequence of events due to respective enzymes in the spectrinase fraction, kinetic analysis of the cleavage processes was performed by MALDI-TOF-MS. The identity of the peptides released upon hydrolysis of Sepharose bead-linked GST-SH3 was assessed after 1, 2, and 3...
Hydrolysis of Spectrin by Malarial Plasmepsin II

Fig. 3. MALDI-TOF-MS identification of cleavage sites in the SH3 amino acid sequence. Sequences 1 and 2, SH3 hydrolysis by recombinant plasmepsin II and the SH3-hydrolyzing activity, respectively. Cleavage sites are indicated by arrows. Sequence 3, kinetic analysis of SH3 hydrolysis by the SH3-hydrolyzing activity. Sites cleaved within 1, 2, and 3 min of incubation are indicated by arrows. The plasmid sequence is underlined.

Recombinant Plasmepsin II Is Able to Hydrolyze Native Spectrin at pH 6.8—Hydrolysis of purified human erythroid spectrin by the Plasmodium acidic spectrinase activity generates three main fragments at 170, 150, and 125 kDa (12). The generation of the 170- and 125-kDa fragments was explained by enzyme(s) cutting within the SH3 motif of the spectrin α-subunit. Because plasmepsin II was found to be part of the acting proteinases in the so-called spectrinase activity, we tested the ability of recombinant plasmepsin II to digest purified native spectrin in order to compare the proteolysis pattern with the one generated by the spectrinase activity. Spectrin from normal erythrocytes was incubated with recombinant plasmepsin II for 1 h, 2 h, and overnight at pH 6.8 and 37 °C. The recombinant enzyme had been preincubated or not for 2 h at pH 5.0 since such conditions induce autoactivation and shortening of the molecule from 43 to 38 kDa (7). Spectrin degradation of the GST-SH3 peptide by 43-kDa recombinant plasmepsin II was analyzed at pH 6.8 as well. Hydrolysis was analyzed by SDS-PAGE and Coomassie Blue staining of the gel. As shown in Fig. 4, the pattern of the hydrolysate was dramatically altered in the presence of 38-kDa plasmepsin II. Densitometric analysis of the gel gave a 84% disappearance of the α-spectrin and a 52% disappearance of the β-spectrin with the 38-kDa form of the enzyme versus almost unchanged α- and β-spectrin amounts in the presence of the 43-kDa form (Fig. 4, lane 3). Also, the amount of protein 4.1 decreased in the presence of 38-kDa plasmepsin II, and actin cleared off within 3 min. From this, we can infer that cleavage of the S∧S site is due to an additional endopeptidase cleaving the fragment -SSINK... VPAVY.

Recombinant Plasmepsin II Selectively Digests Spectrin, Actin, and Protein 4.1 from Erythrocyte Ghosts—The recombinant plasmepsin II activity was tested against ghosts from normal erythrocytes to check if native spectrin, situated within the protein network of the membrane skeleton, would be a substrate for the enzyme and if other proteins from the ghosts would be substrates as well. Erythrocytes were lysed in 5 mM PO<sub>4</sub> buffer (pH 6.8), washed with the same buffer, and incubated overnight at pH 6.8 and 37 °C with the 43- or 38-kDa form of recombinant plasmepsin II. Degradation of the proteins from ghosts was analyzed by SDS-PAGE and Coomassie Blue staining of the gel. As shown in Fig. 5, the pattern of the ghosts was dramatically altered in the presence of 38-kDa plasmepsin II. Densitometric analysis of the gel gave a 84% disappearance of the α-spectrin and a 52% disappearance of the β-spectrin with the 38-kDa form of the enzyme versus almost unchanged α- and β-spectrin amounts in the presence of the 43-kDa form (Fig. 5, lane 2). Also, the amount of protein 4.1 decreased in the presence of 38-kDa plasmepsin II, and actin clearly disappeared.

Immunolabeled Plasmepsin II Can Be Visualized outside the Parasite in Schizont-infected Red Blood Cells—To address the question of the erythrocyte membrane skeleton being a physiologically relevant substrate of plasmepsin II in mature parasite-infected cells, we investigated (by confocal microscopy) the cellular location of the enzyme during the schizont stage. Previous immunoelectron microscopy experiments (5) had shown that plasmepsins are present at the surface of the parasite in trophozoites, but no analysis of plasmepsin location later during parasite intraerythrocytic development was available thus far.

Smears of FcB1-infected red blood cells were fixed in acetone/methanol, and double labeling of cells was performed using rabbit anti-plasmepsin II polyclonal antibodies (serum 737) and mouse anti-MS1 monoclonal antibody (ascites 22-2). Anti-MS1 antibody was used to visualize mature intraerythrocytic parasites via labeling of the parasite membrane. Preservation of red blood cells upon fixation was checked by phase-contrast microscopy. No labeling was observed with non-
immune rabbit serum (data not shown) or with secondary antibodies alone (data not shown).

Images of immunolabeled cells, summed from three 10-nm spaced out sections each, are presented in Fig. 6. Fig. 6A shows an ~40-h-old schizont; although MSP1 labeling is confined to the parasite (panel 3), fleecy labeling with anti-plasmepsin II antibodies can be observed inside and outside the parasite (panel 2). The presence of anti-plasmepsin II antibody labeling in the erythrocyte cytoplasm, although not found in all schizonts, was frequently observed. In Fig. 6B is presented an early segmenter (44–46 h old). Remarkably, plasmepsin II labeling appears to delineate individualizing merozoites, in a comparable way to MSP1 labeling, although MSP1 and plasmepsin II labeling does not totally merge; labeled plasmepsin II appears as a green rim external to the yellow MSP1-delineated membrane.

**DISCUSSION**

Most of the current efforts in understanding the biology of *Plasmodium* aim at elaborating new antimalarial strategies. Protease inhibitors have been successfully employed in well known examples such as AIDS therapy, and identification of target proteases in *Plasmodium* is today part of the antimalarial fight. In this respect, the hemoglobinases, as participants in an essential metabolic pathway, appear to be choice targets. Plasmepsins I and II are both responsible for the initial cleavage of hemoglobin in the digestive vacuole of the intraerythrocytic parasite, but when a specific inhibitor of plasmepsin I (SC-50083) is added to cultures at the trophozoite stage, when hemoglobin is degraded, the parasites have markedly diminished hemozoin production and are killed (IC_{50} = 2 × 10^{-6} M) (5). This indicates that plasmepsin I activity is essential, but also that plasmepsins I and II have distinct hemoglobin-degrading capacities. Moreover, the inability of plasmepsin II to supplant plasmepsin I in hemoglobin degradation raises the question of the exact role of plasmepsin II in this process.

*Plasmepsin II Exhibits Different Substrate Selectivity Compared with Plasmepsin I*—We show here that proteins other than hemoglobin can be substrates of plasmepsin II, namely spectrin, protein 4.1, and actin, all components of the erythrocyte membrane skeleton. Since plasmepsin I was shown to be inactive against a variety of non-globin substrates (2) and was also found to be poorly active on the GST-SH3 substrate, in contrast to plasmepsin II, these findings reinforce the idea that the two enzymes may be devoted to different roles in the parasitized cell. The identification of the five sites cleaved at the two enzymes may be devoted to different roles in the parasitized cell. The identification of the five sites cleaved at the two enzymes may be devoted to different roles in the parasitized cell. The identification of the five sites cleaved at the two enzymes may be devoted to different roles in the parasitized cell.
within the SH3 motif: the F ↓ Q site, deduced from the plas-
mpsine I preference for the phenylalanine at the P1 position, and
the L ↓ L site, listed as a secondary site on hemoglobin (3). The
presence of 32–30-kDa fragment(s) upon hydrolysis of
GST-SH3 by fraction 4 and adjacent functions in Fig. 1 could
thus be attributed to plasmepsin I action. However, fractions in
which plasmepsin II was readily detected compared with plas-
mpsine I (fractions 8–10) displayed higher degradative activ-
ity. This observation suggests that, most probably, recogni-
tion of potential cleavage sites in the SH3 motif by plasmepsines I
and II involves additional parameters such as identity of the
surrounding amino acids and/or conformation of the polypep-
tide chain. In the same way, the number of spectrin sites
actually cleaved by plasmepsin II appears limited since a 20-h
hydrolysis of purified native spectrin at pH 6.8 by recombinant
plasmepsin II produces high molecular mass fragments, al-
though many potential sites are found scattered along the α-
and β-subunit amino acid sequences, predicting the generation
of multiple short sized fragments. Conformational require-
ments might explain why the SH3 motif, whose secondary
structure is different from the repetitive structure of the rest of
the α-chain, is a privileged target for the enzyme. This inter-
pretation would go along with what has been written about the
enzyme specificity depending on the tertiary structure of its
substrate hemoglobin (24).

pH Activity and Maturation of the Recombinant Enzyme—
Recombinant plasmepsin II can digest the SH3 motif of α-spect-
rin, as well as native spectrin, at pH 6.8. In terms of activity of
the enzyme on the GST-SH3 substrate, hydrolysis of the sub-
strate at pH 5.0 is completed within 75 min, whereas at pH 6.8,
primary degradation fragments are visible. Thus, the pH opti-
mum of the enzymatic activity does not seem to differ from
hemoglobin to the GST-SH3 substrate. The activities of puri-
fied native plasmepsins II and I on hemoglobin are known to be
dramatically reduced at pH 6.5. In both cases, ~35% residual
activity is detected (24). Further experiments are needed to
quantify the activity of the recombinant enzyme against the
SH3 motif at pH 6.8 and to compare it with its activity against
hemoglobin at the same pH. An interesting observation is the
enhanced efficiency of recombinant plasmepsin II in degrading
spectrin after it has been shortened to 38 kDa by autoactivation
at pH 5.0. Previous studies had demonstrated that the struc-
tural and kinetic properties of mature recombinant plasmepsin
II and native plasmepsin II were similar (8), but nothing is
known about the activity of the precursor form of recombinant
plasmepsin II since all experiments were performed at pH 5.0.
We observed that within 20 h at pH 6.8, the hydrolysis pattern
of native spectrin, either purified or from ghosts, was greater
when due to the mature form of the enzyme than to the pre-
cursor form. One explanation might be that the enzyme has
slowly autoactivated to the 38-kDa form at pH 6.8. If not, this
might indicate that some of the properties of the enzyme differ
between immature and mature forms in terms of structural
and/or kinetic properties.

Is the in Vitro Degradation of the Host Cell Skeleton by
Plasmepsin II Physiologically Relevant?—Our results raise the
question of the physiological relevance of observations made in
vitro. Indeed, two points must be considered. First, the red
blood cell cytoplasm and plasmepsin II were believed to belong to
distinct cellular compartments; and second, to cleave skeleton
proteins, plasmepsin II would need to act at a pH near neu-
trality in the cytoplasm of the red blood cell. Now, the enzyme
has been found to be active at pH 6.8 against spectrin, actin,
and protein 4.1, which suggests that it could be active in the red
blood cell cytoplasm. Confocal imaging using anti-plasmepsin II
antibodies brought clear evidence of a possible proximity
between the enzyme and membrane skeleton during late para-
site maturation. What has been inferred about plasmepsin I
and plasmepsin II trafficking, largely deduced from the study
of plasmepsin I trafficking, is that plasmepsins are secretory
molecules that are synthesized as type II integral membrane
proteins (21). The proplasmepsins have been detected by im-
munoelectron microscopy at the parasitophorous vacular
membrane (5), and visualization of plasmepsin II outside the
parasite by immunoconfocal microscopy might be due to para-
sitophorous membrane-originated structures extending into
the red blood cell cytoplasm. Plasmepsin II in early segmenters
was found located at the periphery of the parasite, and labeling
appeared higher at this stage than in younger schizonts, even
though massive degradation of hemoglobin is not required
anymore.

Considering the broad substrate selectivity and cleavage site
specificity of plasmepsin II and the large amount as well as the
location of the enzyme in late schizonts, the overall results
presented here strongly suggest that plasmepsin II function in
the parasite intraerythrocytic cycle is not restricted to hemo-
globin degradation. Its involvement in spectrin depletion in
vivo remains to be established, but it is clear that trafficking of
plasmepsins and their respective roles in the infected cell must
be further explored.

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