HEMOGLOBIN-DEGRADING PLASMEPSIN II IS ACTIVE AS A MONOMER

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Running title: Plasmepsin II monomer and hemoglobin degradation

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A family of aspartic proteases called plasmepsins is important for hemoglobin degradation in intraerythrocytic Plasmodium parasites. Plasmepsin II (PM II) is the best-studied member of this family. PM II and its close orthologs and paralogs form homo-dimers with extensive interfaces in all known crystal structures. This raised the question whether the homo-dimer is the functional subunit of plasmepsins in solution. We have used gel filtration chromatography, site-directed mutagenesis and analytical ultracentrifugation to study the oligomeric status of PM II in solution. Our results reveal that PM II exists mainly as a monomer in solution and that the monomer is fully functional for catalysis. A hydrophobic loop at the PM II monomer surface, which would be buried in a PM II dimer, is shown to be essential for the hemoglobin degradation capability of PM II.

Malaria is caused by the apicomplexan protozoan parasite Plasmodium. During its development in host erythrocytes, the parasite engulfs and degrades up to 80% of the host cell hemoglobin (1) and utilizes nutrients released from this catabolic process (2). Hemoglobin degradation is essential for the parasite to survive. Disruption of this process is an attractive drug development strategy.

Hemoglobin degradation occurs in the acidic food vacuole of the parasite (3). Multiple proteases have been identified in the food vacuole, including plasmepsins (4). There is only one food vacuole plasmepsin for most Plasmodium species studied (5,6). The exception is P. falciparum, the causative agent of severe malaria, which has four food vacuole plasmepsins (7). The four proteins share a high degree of homology (60-70% amino acid identity) and are named plasmepsins I, II, IV and HAP. HAP stands for histo-aspartic protease because its catalytic dyad has a histidine instead of one of the two canonical aspartates. Despite this substitution, HAP is believed to be an active protease (7,8). Plasmepsins are involved in the early steps of hemoglobin degradation. They are able to recognize intact hemoglobin and make an initial cleavage in the B helix of the hemoglobin α-chain between Phe33 and Leu34 (9). This cleavage is believed to unravel the globin chain, facilitating subsequent proteolytic cleavages. Recent gene disruption studies have indicated that PM II as well as the other food vacuole plasmepsins are not essential for parasite survival, but each contributes to optimal parasite growth (2,10,11).

The quaternary structure of an enzyme is often critical for its function and regulation. Homo-dimerization is one common quaternary configuration, and it has been implicated in the catalytic activity and stability of some members of the aspartic protease family. For example, HIV-1 protease relies on the formation of the homo-dimer to complete its active site aspartate dyad (12,13). Cathepsin E is a homo-dimer linked by an inter-molecular disulfide bridge (14). The disulfide bridge and thus the dimer, is not essential for cathepsin E activity, but it contributes to the stability of the enzyme (15). Both β-amyloid precursor protein-cleaving enzyme (BACE) and presenilin, the core components of the β- and γ-secretases of amyloid precursor protein respectively, form dimers in the membrane.
It is believed that dimerization is important for BACE to recognize specific substrate motifs (16) and for presenilin to form its inter-molecular diasparyl active site (17). The constraints on variability of dimer interface residues has been exploited in the case of HIV protease to develop potent inhibitors that may be refractory to drug-resistance mutation (18,19).

Multiple X-ray crystal structures of PM II, as well as those of PM IV, Plasmodium vivax and Plasmodium malariae plasmepsins have been determined. Interestingly, all of them are tightly associated homo-dimers (20-26). These structures were obtained using different crystallization conditions and have different crystallographic symmetries. Different ligands are bound, or in one case absent (24). The surface area that is buried in the conserved dimer interface is large, up to ~2500Å² (24). This raised the questions of what quaternary structure plasmepsins have in solution and whether dimerization is necessary for their catalytic function. Because of the high degree of homology among the four P. falciparum food vacuole plasmepsins, especially the conservation of residues at the dimer interface in crystals, it was also possible that plasmepsins could form hetero-dimers in the food vacuole, which might be another means of modulating their catalytic activities in vivo. To explore these possibilities, we studied the quaternary structure of PM II in solution by using gel filtration chromatography, site-directed mutagenesis and analytical ultracentrifugation.

### Experimental Procedures

#### Preparation of Recombinant Proteins

Recombinant PM II was prepared in E. coli as inclusion bodies. The protease was expressed as the mature form, lacking the pro-domain. Recombinant PM II has been shown to have activity comparable to that of native enzyme (27,28). Expression, purification, and refolding were accomplished according to the procedure described in reference (28). Mutant constructs were prepared using QuikChange (Stratagene, La Jolla, CA) with the following primers (complementary primers are not listed): 5'-

CCATTTACATTTATCTTCTACAGGATCTG
CTAATTATGGGTCCC-3' (for D34S)

5'-

GCCAACTGTATTGTATCTAGTGTTACTAGT
GCCATTACTGTACC-3' (for D214S)

5'-

CCATTTACATTTATCTTCTACAGGATCTG
CTAATTATGGGTCCC-3' (for D34A)

5'-

GCCAACTGTATTGTATCTAGTGTTACTAGT
GCCATTACTGTACC-3' (for D214A).

The PM II-Cathepsin E chimera was prepared using a 3-step QuikChange mutagenesis. Step one involved the deletion of residues 241-252 in PM II (primer: 5' - GCAGAATTTAGATGTTATCAAACACAGCA AATTACCAAC -3'). Six amino acids (PVDGEY) of the cathepsin E loop were inserted in step two (primer: 5' - GCAGAATTTAGATGTTATCCGGTTGACGG TGAATCAAACACAGCAATTACCAAC -3'). In step three, 5 additional amino acids (AVECA) of the cathepsin E loop were inserted (primer: 5' - GGTTGACGGTGAATACGCTGTTGAATGCG CTAACAACAGCAATTACCAAC -3'). The resulting chimeric protein contains the amino acid sequence V236I237PVDGEYAVECAN250N251 (lower case numbers correspond to amino acid number of PM II). All mutated sequences were confirmed by PCR sequencing (PNACL, Washington University School of Medicine). A chimera in which the loop from PM II was inserted into cathepsin E was also constructed. This protein failed to refold and could not be analyzed further.

#### Gel Filtration Chromatography

A Waters (Milford, MA) 626 HPLC system and an Amersham (Piscataway, NJ) Hi-load 26/60 Superdex 75pg column were used. The column was equilibrated with 20mM Tris-HCl (pH 8.0), 0.25M NaCl, 10% glycerol. The same buffer was used as eluent at the rate of 1 ml/min. The column was calibrated with Bio-Rad (Hercules, CA) gel filtration molecular weight standards before use. 5 ml of pre-clarified recombinant PM II and its mutants were loaded on the column and the elution was monitored at 280nm. 2 ml...
fractions were collected and pooled as indicated in Fig. 1b.

**Equilibrium Sedimentation**

~ 50 ml of gel filtration-purified PM II was dialyzed against 20mM Tris-HCl (pH 8.0), 0.25 M NaCl overnight at 4°C to remove glycerol. The sample was then concentrated down to 500 µl by ultra-filtration with an Amicon Centrprep YM-30 column (Millipore, Billerica, MA).

Equilibrium sedimentation experiments were performed on a Beckman Coulter (Fullerton, CA) XLA-70 analytical ultracentrifuge equipped with a Ti-50 rotor at the Keck Biophysics Facility at Northwestern University. All experiments were run at 4°C. The samples were sedimented to equilibrium at rotor speeds of 15500 rpm for 35 h, 22300 rpm for 25 h, 29200 rpm for 17 h, 36100 for 10 h and 43000 rpm for 6 h. The equilibrium buffers used were 10mM NaH$_2$PO$_4$, 150mM NaCl, pH 5.2 or 7.2 as noted in the results section. The samples were loaded at four initial concentrations, OD 0.3 and 0.5 at 280 nm, and OD 0.3 and 0.5 at 230 nm. The radial step size was 0.001 cm. Data analysis was performed with Ultrascan 7.0 (http://www.ultrascan.uthscsa.edu) at the University of Texas Health Science Center at San Antonio. The data was fitted to multiple models, including a monomer-dimer equilibrium model, an ideal non-interacting two-component model, and an ideal one-component model.

**Inhibitor Competition Assay**

The enzyme activity assay was modified from the published procedure (28) and the competition assay was based on the work of Tang and colleagues (29). All reactions (in triplicate) were incubated in a 96-well plate at 37°C and the fluorescence was read at the time points of 0, 10, 60, 120 and 180 min. A 96-well fluorescence plate reader adapted to a Cary Eclipse fluorimeter (Varian, Palo Alto, CA) was used. The fluorogenic peptide substrate 2837b, [EDANS-CO-CH$_2$-CH$_2$-CO-ALERMFLSFP-Dap-(DABCYL)OH] (AnaSpec, San Jose, CA) (28), was used at 1 µM. The excitation wavelength was 336 nm and the emission wavelength was 490 nm. Both the excitation and emission slit widths were 10 nm. The reaction buffer was 0.1 M sodium acetate (pH 5.4), 10% glycerol, 0.1% octyl β-D-glucopyranoside (Sigma, St. Louis, MO). When applicable, wild-type PM II was added to the reaction at 0.3 nM. Pepstatin A (Calbiochem) concentrations used were 0 nM, 0.075 nM (molar ratio 1 : 4, inhibitor : enzyme) and 0.3 nM (molar ratio 1 : 1, inhibitor : enzyme) respectively. The mutant proteins were added to the reactions (as indicated in Fig. 3) in 4-fold excess over wild-type PM II based on OD at 280 nm.

**Enzyme Kinetics**

Kinetic parameters of wild-type PM II and the chimera were measured in 0.2 M sodium acetate (pH 5.0), 20% glycerol with substrate 3037b (28) in 400 µl reactions. Averages of three to four measurements were used at each substrate concentration. $K_m$ and $V_{max}$ values were calculated by direct fitting of initial rates using the Michaelis-Menten equation and Kaleidagraph software (Synergy, Reading, PA). Concentrations of enzymes were determined by active-site titration with pepstatin A and the $k_{cat}$ was calculated from $k_{cat} = V_{max} / [E]$. For wild-type PM II, 7.75 nM protease was used in the assays; for the chimera, the concentration was 12.0 nM.

**Hemoglobin and Hemoglobin α-Chain Degradation**

Hemoglobin degradation assays were performed in 0.2 M sodium acetate (pH 5.0), 20% glycerol as described (28). For the hemoglobin degradation, 90 nM protease and 15 µM hemoglobin (based on heme) were used. For the hemoglobin α-chain degradation, similar substrate concentrations but decreased protease (21 nM) were used.

**Results**

**Gel Filtration Chromatography of PM II**

Mature PM II was expressed in *E. coli* as inclusion bodies. The inclusion bodies were refolded and purified to near homogeneity as judged by SDS-PAGE (Fig. 1a, lane 1). The preparation was subjected to one more chromatographic step using gel filtration chromatography. There was no apparent improvement in the homogeneity of the preparation (Fig. 1a, lane 2), but the gel filtration
The observed optical absorbance versus radium position is displayed in Fig. 2 (upper panels, red dots). The data were fitted to different models, including an ideal one-component model, an ideal non-interacting two-component model, and a monomer-dimer equilibrium model. The summary of the results is in Table 1. The fitting to the monomer-dimer model is not shown for the pH 5.2 conditions because the modeling did not converge, suggesting it is a clearly unfitted model. As shown in Table 1, the ideal non-interacting two-component model has the least variance (< 3.0 × 10⁻⁵) in all conditions tested. The other two models have higher variances or cannot be fitted. This result indicates that in each of the conditions tested there are two species with different molecular weights and they are not in equilibrium. For the pepstatin A-bound conditions, the molecular weight for the smaller species is 37.0 kDa at pH 5.2 and 38.8 kDa at pH 7.2. These numbers are close to the theoretical molecular weight of PM II (MW 36.9 kDa) plus pepstatin A (MW 686). The molecular weight for the larger species is about three times the monomeric molecular weight so it is likely due to PM II oligomeric aggregates. At pH 7.2 with no pepstatin A bound, the molecular weight of the smaller species is slightly lower than the theoretical number. This may due to the relatively less compacted structure of the protein when there is no ligand bound and therefore the buoyancy would be larger. At pH 5.2 with no pepstatin A bound, most PM II has been degraded over the course of the equilibrium sedimentation as we predicted based on preliminary experiments. The molecular weight of 7.2 kDa could be due to degraded PM II fragments.

The simulated curves of the best-fit models are displayed in Fig. 2 (upper panels, blue lines) and the corresponding residues for observed data points are also graphed in Fig. 2 (bottom panels). For all the conditions, the residues are centered at zero and randomly distributed with small variance. This indicates that the non-interacting two-component model faithfully represents the oligomeric status of PM II in solution.

From the molecular weight – optical density graphs (Fig. 3), it can be seen that the formation of the oligomeric PM II from monomers is an irreversible process. When approaching the

Analytical Ultracentrifugation

In order to investigate this further, we subjected the active fraction (pooled peak 2) to analytical ultracentrifugation. Equilibrium sedimentation data were collected and a global equilibrium analysis was performed. This method has the advantage of being able to accurately determine molecular weight independent of molecule shape and matrix-protein interactions, and to access the distribution of different molecular weight species in the system. In particular, a monomer-dimer equilibrium can be detected, if it exists. We did the analysis at pH 7.2, at which PM II is catalytically slow, and at pH 5.2, at which PM II has optimal activity and is close to its physiological environment in the food vacuole. Because it is known that ligand binding changes enzyme shape and may promote inter-molecular interactions, we did the analysis in the presence of pepstatin A (protein : inhibitor, 1 : 2 molar ratio) and compared the results to those without it. At pH 5.2, pepstatin A proved essential because in its absence there was significant self-degradation of PM II at the high protein concentration required for sedimentation analysis, even when the analysis was done at 4 °C. Multiple rotor speeds and protein loading concentrations were used (details in experimental section). The global analysis approach removes the concern that a set of data can be fitted to an incorrect model as may happen in the case of sedimentation velocity analysis or equilibrium sedimentation analysis using few conditions.
same protein concentration at different rotor speeds and from different loading concentrations, aggregates with different oligomeric status are formed. This is consistent with the results of gel filtration chromatography (Fig. 1, b and c). There, PM II lost its enzymatic activity after the formation of soluble oligomeric aggregates and activity could not be restored by dilution.

**Competition with Active-site Mutants of PM II**

A number of eukaryotic aspartic proteases rely on the formation of homo-dimers to gain catalytic activity (12,13,17). If PM II were to form dimers in solution and rely on the dimer for catalysis, replacement of one of the monomers with an active-site mutant should abolish the wild-type PM II activity. Alternatively, if the monomer is the catalytic unit, mixing wild-type PM II with active-site mutant would not affect the wild-type activity. We mutated the PM II catalytic aspartates (D34A, D34S, D214A and D34S/D214S). These mutations abolished proteolytic activity (Fig 4, red lines). To assess correct refolding (29), we mixed wild-type and mutant PM II and assessed ability of mutant enzymes to bind pepstatin A competitively and thus relieve pepstatin inhibition of wild-type activity. As shown in Fig. 4, D214A, D34S/D214S and to a lesser extent D34S could competitively bind pepstatin A and restore wild-type PM II activity when the mutant enzyme was added in excess. This is an indication that the three mutants had refolded correctly. In contrast, D34A was not able to bind pepstatin A, perhaps reflecting improper enzyme folding. In the absence of pepstatin A, the proteolytic activity of wild-type PM II was not affected by addition of excess mutant enzyme (Fig 4, blue lines). This suggests that the catalytic unit of PM II is the monomer, rather than the dimer.

**Enzymatic Activity of PM II-cathepsin E chimera**

Human hemoglobin is initially cleaved by PM II between α-Phe33 and Leu34. This peptide bond is buried within an α helix in the tightly packed, hydrophobic hemoglobin molecule. It is an intriguing question as to how PM II active site residues access the Phe33-Leu34 peptide bond and make the initial cleavage. One possible mechanism is that PM II may be a hemoglobin "denaturase", using residues outside the active site to interact with hemoglobin and make the Phe33-Leu34 peptide bond accessible (28). Candidate residues that might interact with the hydrophobic hemoglobin surface residues are found among a hydrophobic stretch (239-248, VPFLPFYVTLC) conserved among food vacuole plasmepsins (28). They form a loop and are buried at the dimer interface in PM II crystal structures, but would be exposed when PM II exists as a monomer in solution (Fig. 5). Interestingly, cathepsin E, a mammalian ortholog of the plasmepsins that can cleave hemoglobin only after denaturation, has a different sequence (PVDGEYAVECA) at the corresponding site. If the hydrophobic loop of PM II is essential for its hemoglobinase activity, replacement of the loop by the corresponding cathepsin E sequence would be predicted to abolish the hemoglobin degradation activity of PM II. On the other hand, the degradation of small peptide substrate should not be affected by this replacement if the mutation doesn't affect the refolding of the enzyme adversely.

We constructed a chimeric aspartic protease based on PM II, with the hydrophobic loop replaced with the sequence from cathepsin E. As shown in Figure 6 panel A, peptide-cleavage kinetics of the PM II-cathepsin E chimera was comparable to those of wild-type PM II. This suggests that the distal loop does not contribute to the mechanism of peptide-bond hydrolysis. The chimera was also capable of cleaving isolated hemoglobin α-chains, which are less tightly folded than in the tetramer (Fig. 6 panel B). However, the chimera was dramatically compromised in its ability to cleave intact hemoglobin (Fig. 6 panels C and D). The data suggest that this loop is crucial for the ability of plasmepsins to degrade native hemoglobin. Addition of the PM II D34S/D214S double mutant in excess had no effect on hemoglobin degradation (data not shown). This suggests that dimer formation is not critical for efficient hemoglobin degradation and further supports the notion that dimer formation does not happen in solution, since the loop is buried in the crystallographic dimer.

**Discussion**

The existence of multimers in a crystal structure is often an indication that a similar complex can form in solution. This is not a
universal rule and certain examples exist where complexes formed in a particular crystallization condition do not exist in solution. In the current case, PM II is a dimer in all known crystal structures. Nevertheless, there is no detectable dimer in solution, using conditions under which the enzyme is active. Our evidence for this includes gel filtration fractionation and sedimentation equilibrium ultracentrifugation. Some aggregates formed irreversibly in these analyses, but the rest was monomer in each case, with and without ligand. Consistent with this finding, activity appears to reside in the monomer since the monomer gel filtration peak was fully active and since catalytically dead enzyme could not diminish activity of wild-type enzyme by heterodimerization.

This finding has important implications for understanding how PM II works. PM II is capable of cleaving hemoglobin at acidic pH between Phe33 and Leu34. Cleavage at this position facilitates further unfolding of hemoglobin and increases its accessibility to other food vacuole proteases (4). In the available neutral pH structures of hemoglobin, Phe33 and Leu34 are buried in the B-helix of the α-chain and are not accessible to solvent. It is not established how low pH affects the structure of hemoglobin, particularly at the B-helix of the alpha-chain. However, proteases such as cathepsin E can not cleave hemoglobin at pH 5 even though they readily cleave isolated (less tightly folded) α-chains well (28), suggesting limited availability in tetrameric hemoglobin. We propose that the loop residues that contribute to the highly conserved dimer interface observed in all available plasmepsin crystal structures may comprise a protein-protein interaction motif. When plasmepsin is the sole molecule in solution, dimers can form under molecularly crowded conditions (crystallization). However, in the context of the food vacuole, the hydrophobic loop of the plasmepsin monomer could interact with and possibly intercalate into the hemoglobin substrate. This interaction may open the B-helix of hemoglobin, exposing the Phe33-Leu34 peptide bond for hydrolysis.

The action of the plasmepsin hydrophobic loop differs from the recently reported hemoglobin recognition motif in falcipains, the other food vacuole hemoglobinase family (30-32). The latter loop confers ability of falcipains to bind and cleave hemoglobin or denatured globin. Its function is presumably in substrate recognition. In contrast, the plasmepsin loop appears to be involved in substrate accessibility for catalysis. Perhaps it works by stretching the globin helix to expose the scissile bond. In this regard it is notable that the B helix of the alpha chain of hemoglobin is a weak alpha helix, possessing two destabilizing glycines in the helix. Most proteases are unable to cleave intact hemoglobin until substrate denaturation has occurred. Thus, it will be interesting to further explore the mechanism of this hemoglobinase.

References


Footnotes

* These authors contributed equally to this work.
# Current address: Oregon Health & Science University, Portland, Oregon.
The abbreviations used are: PM II: plasmepsin II; OD: optical density.
Acknowledgements

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Figure Legends

Figure 1. Analysis of recombinant PM II.
(A) Electrophoresis of recombinant PM II on 12% SDS-PAGE. The gel was stained with Coomassie blue. Lane 1, sample before gel filtration chromatography. Lane 2, pooled and concentrated peak 2 fractions from the gel filtration column. Size of molecular weight markers (in kDa) is indicated on the left. The impurity migrating at 25 kDa is estimated to constitute less than 1% of total protein. (B) Gel filtration chromatography of PM II. 2 min fractions were collected and pooled as indicated (numbers above the peaks). The arrows denote MW markers (from left to right: bovine thyroglobulin, 670 kDa; bovine γ-globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa) used for calibration. (C) Enzymatic activity of pooled peaks from (B). 5 µl samples from peak 1 and peak 2 in (B) were diluted 100 times and their enzymatic activity assayed as described in experimental procedures.

Figure 2. Equilibrium sedimentation analysis: optical density – radium graph.
(A) pH 7.2, plus pepstatin A; (B) pH 7.2, no pepstatin A; (C) pH 5.2, plus pepstatin A; (D) pH 5.2, no pepstatin A. Upper panels: observed optical density distribution along the radium (red dots) and simulated curves of the best-fit models (blue lines). Bottom panels: residue optical density of the fitted lines to the observed data. Between 12 to 20 runs with multiple rotor speeds and initial protein loading concentrations were performed for each condition. The model fitting and curve simulation was done with UltraScan 7.0 software.

Figure 3. Equilibrium sedimentation analysis: molecular weight – optical density graph.
(A) pH 7.2, plus pepstatin A; (B) pH 7.2, no pepstatin A; (C) pH 5.2, plus pepstatin A; (D) pH 5.2, no pepstatin A. The analysis was done with UltraScan 7.0.

Figure 4. Competition with PM II active site mutants.
Triplicate reactions were incubated in a 96-well plate and fluorescence was monitored over time. Solid symbols: 0.3 nM wild-type PM II in incubation with no (blue dots), 0.075 nM (black squares), and 0.3 nM (black triangles) pepstatin A; Red circles: no wild-type PM II and no pepstatin A added. The active site mutant PM II competitors added to each reaction at 1.2 nM are denoted in the corner of each panel. Error bars represent standard deviation of triplicate wells.

Figure 5. PM II model.
Model of two proximal PM II monomers (blue and gold) illustrating the dimer based on the coordinates of the crystal structure with PDB code 1LEE. The inhibitor (RS367) is indicated in grey. Residues of the hydrophobic loop (239-248) at the dimeric interface are highlighted in red.

Figure 6. Enzymatic properties of PM II-cathepsin E chimera in comparison with wild-type PM II.
(A) Kinetics of cleavage of peptide substrate 3037b. Circles and solid line mark wild-type PM II. Squares and dashed line mark the chimera. The curves were simulated by the Michaelis-Menton equation. Error bars indicate standard deviations of 3-5 measurements. The thin error bars correspond to the wild-type data and the thick error bars correspond to the chimera data. For the wild-type, $K_m = 4.96 \pm 0.23 \mu M$ and $k_{cat} = 0.80 \text{ sec}^{-1}$; For the chimera, $K_m = 5.76 \pm 0.36 \mu M$, $k_{cat} = 0.59 \text{ sec}^{-1}$.
(B) Degradation of hemoglobin α-chain by wild-type PM II (Wt) or PM II-cathepsin E chimera (Chimera). α-chain was incubated at 37°C with equal amount of wild-type or chimera in 0.2 M sodium acetate, pH 5.0, 20% glycerol. Aliquots were removed at the times indicated, and reactions were quenched by diluting with SDS-PAGE sample buffer and boiling for 3 min. Gel electrophoresis was performed on 15% SDS-PAGE with Coomassie blue staining. Ctrl denotes no protease added.

(C) Degradation of hemoglobin by wild-type PM II (Wt) or PM II-cathepsin E chimera (Chimera). Hemoglobin was incubated for 23 hours with protease as in (B). Gel electrophoresis was performed on 12% SDS-PAGE and the procedure is same as (B).

(D) Quantification of hemoglobin cleavage by wild-type PM II (solid columns) or PM II-cathepsin E chimera (open columns). Hemoglobin was incubated with 2.4 nM protease as in (C). The degradation was analyzed by 12% SDS-PAGE and quantified by densitometry. Reactions were performed in duplicate and error bars indicate standard deviations.
Table 1. Summary of global equilibrium analysis

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<th>MW_B (kD)</th>
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*: A states for ideal one component model; A, B states for ideal, two non-interacting component model; 2A ⇌ A₂ states for monomer-dimer equilibrium model. K_a: association constant.
Figure 1

A

B

C

Absorbance 280nm

Elution time (min)

Fluorescence units

Time (min)
Figure 2

A  B  C  D

Residue  Optical density

\[ r^2 - r_0^2 \text{ (cm)} \]
Figure 4

Fluorescence units

Time (min)

None
D34A
D34S
D214A
D34S/D214S
Hemoglobin-degrading plasmpsin II is active as a monomer
Jun Liu, Eva S. Istvan and Daniel E. Goldberg

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