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Hemoglobin Degradation

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Abstract Hemoglobin degradation by *Plasmodium* is a massive catabolic process within the parasite food vacuole that is important for the organism's survival in its host erythrocyte. A proteolytic pathway is responsible for generating amino acids from

hemoglobin. Each of the enzymes involved has its own peculiarities to be exploited for development of antimalarial agents that will starve the parasite or result in build-up of toxic intermediates. There are a number of unanswered questions concerning the cell biology, biochemistry and metabolic roles of this crucial pathway.

Abbreviations

RBC	Red blood cell
HAP	Histo-aspartic protease
MPP	Mitochondrial processing peptidase
ER	Endoplasmic reticulum

1 Introduction

Malaria parasites in the bloodstream reside within host erythrocytes (red blood cells; RBCs). About 95% of the soluble RBC protein is hemoglobin, which is present at a concentration of 340 mg/ml. This serves as a rich nutrient source for parasite metabolism. An estimated 75% of the hemoglobin is consumed by *Plasmodium falciparum* during its brief intraerythrocytic stay [1–3]. Thus, hemoglobin degradation is a massive and rapid catabolic process. A number of the enzymes involved have been studied in detail and have some unusual features. A description of these enzymes, their roles in hemoglobin degradation, their biosynthesis and targeting, forms the basis for this review.

1.1 Purpose of Hemoglobin Degradation

Plasmodium parasites utilize hemoglobin as an amino acid source for protein synthesis. The evidence for this is that amino acids from radiolabeled hemoglobin get incorporated into parasite proteins [4, 5], and that despite limited ability for de novo amino acid synthesis, *P. falciparum* can survive in medium supplying just five amino acids that are in limited supply or absent from hemoglobin [6, 7]. Use of amino acids for growth appears to be important, because parasites grown in the five amino acid medium are more sensitive to hemoglobin degradation inhibitors than those grown in full medium [7].

Amino acids can also be used as an energy source, though the metabolic significance of this is unknown. Hemoglobin degradation appears to have nonanabolic roles as well. A significant portion of amino acids released from hemoglobin is excreted by the intraerythrocytic parasite [8,9], and it has been proposed that the parasite is making room for itself in its host cell [9,10], or that it is controlling RBC osmotic stability [11]. Even in medium containing all 20

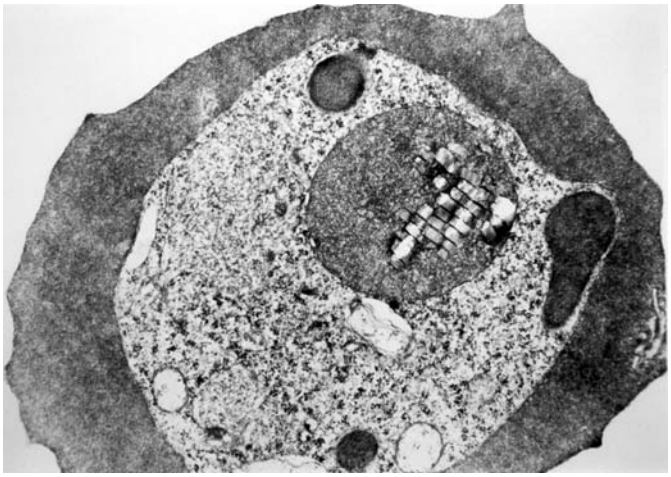

Fig. 1 Transmission electron micrograph of a *P. falciparum* trophozoite within an erythrocyte. At the *top* of the field, a cytostome is seen ingesting hemoglobin for delivery to the food vacuole, which is already filled with hemozoin crystals

amino acids, hemoglobin degradation inhibitors are effective, suggesting that exogenous amino acid supplementation cannot fully override hemoglobin proteolysis blockade.

1.2 Site of Degradation

Plasmodium ingests hemoglobin from the host cell through an opening called the cytostome, an invagination of the parasitophorous vacuolar and parasite plasma membranes. Hemoglobin (and other RBC content) is transported to the acidic food vacuole for degradation (Fig. 1). Little is known about the cell biology of hemoglobin ingestion. It has been hypothesized that hemoglobin degradation may start in transport vesicles before delivery to the vacuole [12]. There is no solid evidence to support this interesting possibility. Clearly the food vacuole is a major site of hemoglobin degradation. Its function has been reviewed in detail [13].

1.3 Degradation Pathway

A multitude of proteases have been localized to the food vacuole and proposed to play a role in hemoglobin degradation. These include a group of aspartic proteases called plasmepsins, a group of cysteine proteases called falcipains,

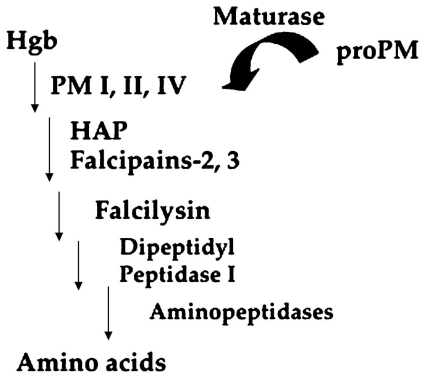

Fig. 2 Proposed hemoglobin degradation pathway. *Hgb*, hemoglobin; *PM*, plasmepsin

a metalloprotease called falcilysin, and at least one dipeptidylpeptidase I. Aminopeptidases are thought to be involved, but their site of action has not been established. There is evidence (discussed below) that the degradative enzymes function in a semi-ordered pathway (Fig. 2), with plasmepsins making the initial cleavage in intact hemoglobin, followed by secondary cleavages by plasmepsins and falcipains. Falcilysin appears to recognize only short peptides generated by upstream enzymes, while the dipeptidylpeptidases and aminopeptidases are presumed to function most efficiently in terminal degradation/amino acid release. An alternative view of the pathway has falcipains participating in the initial cleavage (see Sect. 3). It is clear that inhibitors of multiple classes of protease involved in hemoglobin degradation kill the parasites in culture and/or in animal models and therefore merit development as antimalarial drug targets.

Early in the degradative pathway, heme is released and is detoxified by assembly in a crystalline array called hemozoin. Antimalarial 4-aminoquinolines appear to function by disrupting this sequestration, leading to an accumulation of toxic heme products. This topic is discussed in the chapter by Bray et al. and Scholl et al., this volume.

2 Plasmepsins

2.1 Genomics

The plasmepsins are a group of aspartic proteases whose members were first discovered and purified by following their hemoglobinase activities [7, 14].

P. falciparum has 10 plasmepsins in its genome [15]. Other human and animal parasite species have orthologs of IV–X [16]. In *P. falciparum* or a near ancestor, IV appears to have undergone multiple gene duplications, giving rise to I, II and HAP (histo-aspartic protease, formerly plasmepsin III, see below). Plasmepsins I, II, IV and HAP genes are clustered on chromosome 14 and encode proteins that have 60%–70% amino acid identity. In contrast, these sequences are quite distant from those of the other (non-*Plasmodium*) plasmepsins (10%–20% identity).

2.2 Temporal and Spatial Location

Plasmepsins I, II, IV and HAP are all expressed in intraerythrocytic parasites and are located in the food vacuole [17]. Transcripts for plasmepsins I and IV are detected early in intraerythrocytic development (ring stage) while those for plasmepsin II and HAP are detected later (trophozoite stage) [18, 19]. Protein levels and even plasmepsin biosynthesis persist over a wider swathe of the intraerythrocytic cycle [17, 18]. How these expression patterns relate to function of the different plasmepsins has not been established. Plasmepsins II and IV have been shown to cleave spectrin in vitro and could therefore play an additional role later in intraerythrocytic development [20, 21]. The other six plasmepsins are expressed in other stages or are located in other parts of the parasite.

2.3 Specificity

All four food vacuole plasmepsins have some capacity to cleave native hemoglobin in vitro, though plasmepsin I may be the best under the conditions studied [17, 22]. It needs to be pointed out that crude gel assays do not allow real quantification or kinetic determination of hemoglobin cleavage. Specificity of native hemoglobin cleavage by plasmepsin I has been studied in detail [14, 22]. Initial cleavage occurs between 33Phe and 34Leu on the alpha chain of hemoglobin. Following this cleavage several other cleavages can occur. In 'less native' alpha globin preparations, initial cleavage at multiple sites occurs, giving rise to the model that the initial 33–34 cleavage in intact hemoglobin unravels the hemoglobin molecule so that other sites become accessible. Plasmepsin II is also capable of cleaving at the alpha 33–34 peptide bond but its secondary cleavage sites differ from those of plasmepsin I [22]. Plasmepsin IV has also been shown to make an early cleavage at 33–34 [21].

A number of techniques has been used to study the cleavage of synthetic peptides by plasmepsins. Using chromogenic substrates substituted with

a series of residues one position at a time, plasmepsin II was found to prefer hydrophobic residues at P3, P2 and P3' [23] (nonprime numbering starts N terminal to the cleavage and counts upstream; prime side numbering starts C terminal to the cleavage and proceeds downstream). Proline was preferred at P4 and alanine at P2'. In these studies, basic amino acids were not tolerated in P3. This finding is inconsistent with the fact that there is an arginine at P3 of the initial cleavage site of hemoglobin. Also, fluorogenic peptides with P3 arginine are cleaved well [24]. This suggests that neighboring amino acids can influence specificity in native substrate hemoglobin, and that higher order structural features may shape specificity.

Combinatorial peptide inhibitor libraries have also been used to probe plasmepsin II specificity, and a preference for P2 branched amino acids fits nicely with the hemoglobin cleavage data [25]. Random peptide libraries have been used to probe prime side specificity [26]. The data reveal a strong preference for leucine in P1', as is found with native hemoglobin as substrate. P1' specificity could not be assessed in the chromogenic substrate assays because this residue is fixed as a nitrophenol reporter moiety.

Plasmepsins I, IV, as well as the IV orthologs from *P. vivax* and *P. malariae* have been studied using chromogenic substrates [27]. These studies emphasize differences at the P2 position. Interestingly, when recombinant plasmepsin II was made with nine amino acid substitutions to recapitulate the plasmepsin I active site surface, the specificity for hemoglobin, peptides and inhibitors remained the same as for plasmepsin II [26]. This suggested that active site geometry is more important than amino acid functionality in explaining differences in specificity between these homologous enzymes, and may be influenced by distal amino acids.

2.4 Structure and Mechanism

Plasmepsins are made in the cell as inactive proenzymes. A convertase cleaves them to generate the mature enzyme (see below). Recombinant proplasmepsins II and IV and to a limited extent proHAP are able to autoactivate by cleaving themselves fortuitously at a site near the natural cleavage site [21, 24, 28, 29]. Recombinant proplasmepsin I can autoactivate if a construct with a mutation in the propiece is made [30]. The structure of autoactivated recombinant *P. falciparum* plasmepsin II has been solved [31]. More recently a structure of *P. vivax* plasmepsin IV has been elucidated and is quite similar [32]. The corresponding proenzyme structures have also been determined [32, 33]. A structure of *P. falciparum* plasmepsin IV has been deposited in the protein database but an analysis has not been published.

The plasmepsin II structure reveals a typical two-lobe eukaryotic aspartic protease fold [31]. Indeed the plasmepsins are about 30% identical to mammalian aspartic proteases such as cathepsin D and renin. The inhibitor pepstatin sits in the active site of plasmepsin II and mammalian homologs similarly, but both ends of the molecule show conformational differences when comparing host and parasite enzymes. The thermodynamics of pepstatin binding also differ [34]. These results suggest that selective inhibitors are feasible, and indeed this has been confirmed experimentally (see Sect. 2.5).

The plasmepsins mentioned above are dimeric in the crystalline state and have extensive subunit interfaces [35]. It appears that these enzymes are dimeric in solution as well and that dimerization is important for activity and specificity [36]. This feature may be exploitable for development of selective chemotherapy.

The plasmepsins appear to function as typical aspartic proteases, using two aspartates for acid-base catalytic activation of a water molecule to promote peptide bond hydrolysis. This may also be the case for HAP, the paralog that has a histidine in place of the first catalytic aspartate [17]. Studies with peptide substrates show that HAP has kinetics that are similar to those of other plasmepsins and that it is potently inhibited by pepstatin, a transition-state mimic that forms hydrogen bonds with both aspartates in its action on aspartic proteases.

The proforms of the plasmepsins are quite unusual [32, 33]. The propiece forces open the active site and distorts it so that catalytic activity is prevented. Upon maturation (see below), extensive N-terminal refolding and rotation bring the catalytic machinery to the appropriate geometry for substrate hydrolysis to occur.

2.5 Inhibitors

A variety of inhibitors have been developed to target the plasmepsins. A comprehensive discussion of these compounds and of drug development efforts is beyond the scope of this review and is covered elsewhere [37, 38]. Some general comments will be made here. Many of the compounds generated so far are quite potent against isolated enzymes and some are quite selective for parasite over host enzymes, but they have insufficient activity against cultured parasites. The most active agents have mid to high nanomolar culture potencies[30, 37, 39, 40] and attempts to improve their potency have not yet been successful. Two reasonable explanations for this are that inhibitors have poor bioavailability or that they are potent against only a subset of nonessential plasmepsins. Most rational and combinatorial drug efforts have focused on plasmepsin II because it has been the plasmepsin for which substantial

quantities of recombinant enzyme can be generated and for which a crystal structure has been determined. Unfortunately, drug studies and recent gene knockout experiments suggest that plasmepsin II is not an essential gene, nor are the other food vacuole plasmepsins [41, 42]. Indeed it is possible that the redundant function of plasmepsins is extensive enough that an inhibitor must block most or all of the food vacuole plasmepsins to kill parasites. An attempt to develop adaptive inhibitors that bind the conserved portions of the plasmepsin active site and can rotate an asymmetric functional group to interact well with the unconserved part of the substrate binding pocket, appears promising [29].

3
Falcipains

3.1
Genomics

The falcipains are papain family cysteine proteases initially identified by their role in hemoglobin degradation [22, 43, 44]. *P. falciparum* has four falcipain genes, 1, 2, 3 and 2'—a gene that is 99% identical to 2 in the mature protein but is quite divergent in the propiece. All except falcipain-1 are clustered on chromosome 11. Falcipains-2 and 3 share 53% identity, while falcipain-1 is more distantly related [45]. Rodent falcipain homologs have been characterized [46–48], though a comprehensive evolutionary analysis has not yet been carried out.

3.2
Temporal and Spatial Location

Falcipains-2 and 3 are expressed in intraerythrocytic trophozoites and schizonts [45,49]. Falcipain-3 may turn on slightly later than 2. Antisera to falcipain-2 have localized this protein to the food vacuole as well as to regions outside the food vacuole [49,50]. A role in cleavage of ankyrin during host cell exit has been proposed [50,51]. Falcipain-1 appears to be located in an apical organelle of late-stage parasites and may play a role in invasion [52].

3.3
Specificity

Falcipains-2 and 3 prefer leucine at the P2 position of synthetic peptide substrates [45,49]. Falcipain-3 catalysis is enhanced by valine at P3. It is the subject of some debate whether the falcipains are capable of cleaving native

hemoglobin. Little hemoglobin cleavage is detected unless a reducing agent is added to the reaction [53]. Reducing agents denature hemoglobin [54], though under mild reducing conditions where hemoglobin denaturation cannot be detected spectrophotometrically, some hemoglobin cleavage is seen [53]. The possibility of undetected partial denaturation has not been excluded. Whether or not the falcipains have a small amount of native hemoglobin-degrading activity, they clearly work much better on denatured substrate [22, 53]. Falcipain inhibitor treatment of cultured parasites leads to hemoglobin accumulation in the food vacuole after prolonged incubation; this has been argued as being in favor of an initial role for falcipains in hemoglobin degradation [43, 55]. An alternative interpretation is that this is an indirect effect since shorter treatment does not yield hemoglobin accumulation but does allow heme release from hemoglobin, an action that is blocked by plasmepsin inhibitors [56–58]. A possible mechanism for the indirect effect of falcipain inhibitors has been proposed. Accumulation of peptide fragments from the action of upstream enzymes (plasmepsins) leads to the osmotic swelling of the food vacuole seen with cysteine protease inhibitor treatment, leading to food vacuole dysfunction and hemoglobin accumulation [59].

3.4 Structure and Mechanism

No crystal structure has been determined for the falcipains, though homology modeling based on other cysteine proteases such as papain has been performed [60]. The enzymes are blocked by standard cysteine protease inhibitors and appear to have a typical papain-family thiol protease mechanism of action.

3.5 Inhibitors

A variety of potent falcipain inhibitors in different classes have been identified and/or developed. These efforts are reviewed in detail elsewhere [61]. A few general comments will be made here. Many are potent against cultured parasites and some work in rodent malaria models. There is synergism with plasmepsin inhibitors in the test tube, in culture and in the rodent model [22, 62, 63]. Certain falcipain inhibitors show substantial promise and are under development by the Medicines for Malaria Venture [64]. It is still unclear which of the falcipains need to be inhibited to kill the parasite. A gene disruption of falcipain-2 grows normally, but is more sensitive to aspartic protease inhibitors [65].

4
Falcilysin

4.1
Genomics

Falcilysin is an M16 family metalloprotease identified in a search for a food vacuole activity that could cleave hemoglobin fragments at polar residues [66]. It is a single copy gene on falciparum chromosome 14. There are a number of other metalloproteases in the falciparum genome, all quite distantly related [67]. There are several other M16 family members; one is an apicoplast enzyme [68] and several others appear to have mitochondrial targeting sequences. Falcilysin may be the only food vacuole metalloprotease, though the possibility that others reside there has not been excluded.

4.2
Temporal and Spatial Location

Falcilysin is expressed in trophozoites and schizonts, similar to other globinases [69]. Immunolocalization studies show that the enzyme is in the food vacuole [70]. It is also located in endoplasmic reticulum (ER)-like membranes. Whether it has a separate function there has not been established (see next section).

4.3
Specificity

Falcilysin does not degrade native or denatured hemoglobin but recognizes hemoglobin peptides of 10–20 amino acids [66]. Its specificity has been studied in detail using a random peptide library. These experiments have shown that the enzyme has quite different specificities at acidic and neutral pH [70]. With some substrates the enzyme appears to be a neutral-to-alkaline protease, while with others it is clearly an acidic protease. This finding makes a second function outside the food vacuole seem entirely possible.

4.4
Structure and Mechanism

The crystal structure of falcilysin has not yet been solved. The structure of one M16 family member that has 20% identity with falcilysin, mitochondrial processing peptidase (MPP), is known but is a dimeric enzyme with its catalytic pocket at the subunit interface [71]. This is unlikely to be the case for falcilysin, which has poor homology with MPP in the dimer interface sequence

and is a larger protein with its catalytic residues near the N terminus. The function of the rest of the molecule is unknown. How falcilysin achieves its dual pH-dependent specificity remains to be determined.

4.5 Inhibitors

Metal chelators block the activity of falcilysin. No selective agents have yet been identified. A preliminary attempt to disrupt the falcilysin gene by homologous recombination using positive/negative selection was unsuccessful. This raises the possibility that falcilysin is essential for parasite viability and therefore that the enzyme may be a good drug target.

5 Other Proteases

The *P. falciparum* genome contains several genes encoding proteases that may be located in the food vacuole and that may have a role in hemoglobin degradation [67]. There are three dipeptidyl peptidase-1 homologs, at least one of which is in the food vacuole. There are oligopeptidases that might function in the food vacuole—in other systems these can be degradative enzymes. There is a number of aminopeptidases [67, 72, 73]; one has been localized, in part, to a rim around the food vacuole [74].

Extracts of food vacuoles were capable of breaking down hemoglobin into small peptides [75]. No free amino acids were detected. This raised the possibility that the food vacuole generates peptides and exports them for terminal degradation by cytosolic aminopeptidases. An alternative explanation is that the food vacuole does generate amino acids in vivo but that the downstream enzymes were not active in the food vacuole extracts under the conditions used. This is not an entirely academic issue because in the first case, peptide transporters would be required at the food vacuole membrane, while in the latter case, amino acid transporters would be needed. Both classes of transporter exist in the genome and could be interesting drug targets.

6 Biosynthesis

Biosynthesis of the plasmepsins has been studied most extensively. The plasmepsins are synthesized in the ER as type II integral membrane proteins, anchored by a hydrophobic stretch in the proregion [18]. Antibody [7] and

green fluorescent protein tagging [76] studies have revealed that the proplasmepsins go through the secretory pathway to the surface of the parasite, perhaps directly to the cytostome. From there, the proplasmepsins are internalized along with their eventual substrate hemoglobin. Targeting signals have not yet been identified. At some point in the delivery pathway, most likely upon reaching the food vacuole, the plasmepsins are cleaved from the membrane by an acid convertase, resulting in activation [18, 76, 77]. Cleavage occurs after a conserved Pro-Gly motif in the proregion and is mediated by an ALLN-sensitive enzyme with acid pH-optimal activity [18, 77]. This processing protease has not yet been isolated.

Falcipains also have substantial propieces and are activated by cleavage [45, 49]. Their biosynthesis has not been extensively studied. Recombinant falcipains-2 and 3 can be generated by activation of the proenzymes, and mature profalcipain-2 can be folded without the prodomain by inclusion in cis or in trans of a small chaperone peptide found as an N-terminal extension to the mature protease [78, 79].

Falcilysin does not have a propiece, but rather is synthesized as the mature form [69]. It is a peripheral membrane protein and might be targeted by association with another protein. Its trafficking is brefeldin A-insensitive.

7 Unanswered Questions

A number of issues remain to be answered in the field. Among them are the following:

- Why does the parasite degrade hemoglobin? For nutrients? For osmolar balance? To make room in its host cell?
- How does the cytostome form and function in hemoglobin ingestion?
- How does a protease recognize and cleave a specific peptide bond in a large, folded protein substrate? Specifically, how do the plasmepsins recognize the B helix on the alpha chain of hemoglobin and access a peptide bond that is wound up in the helix?
- Does having a substantial complement of proteases improve the efficiency of degradation compared with having fewer, less specific proteases?
- What are the proteases involved in downstream steps of proteolysis?
- Does the food vacuole generate free amino acids or does it export peptides for terminal degradation in the cytosol?
- How are the hemoglobin-degrading enzymes targeted to the food vacuole?

- What is the proplasmepsin maturase that activates the hemoglobin degradation pathway?
- How can we better exploit the eccentricities of this pathway to design potent and selective inhibitors?

It is worth continued effort to understand this important metabolic process. Biochemical, genetic and chemical studies have the potential to lead us to new antimalarial chemotherapies based on interference with the hemoglobin degradation pathway.

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