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Review

Structural studies of vacuolar plasmepsins ☆

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ABSTRACT

Plasmepsins (PMs) are pepsin-like aspartic proteases present in different species of parasite *Plasmodium*. Four *Plasmodium* spp. (*P. vivax, P. ovale, P. malariae,* and the most lethal *P. falciparum*) are mainly responsible for causing human malaria that affects millions worldwide. Due to the complexity and rate of parasite mutation coupled with regional variations, and the emergence of *P. falciparum* strains which are resistant to antimalarial agents such as chloroquine and sulfadoxine/pyrimethamine, there is constant pressure to find new and lasting chemotherapeutic drug therapies. Since many proteases represent therapeutic targets and PMs have been shown to play an important role in the survival of parasite, these enzymes have recently been identified as promising targets for the development of novel antimalarial drugs. The genome of *P. falciparum* encodes 10 PMs (PMI, PMII, PMIV-X and histo-aspartic protease (HAP)), 4 of which (PMI, PMII, PMIV and HAP) reside within the food vacuole, are directly involved in degradation of human hemoglobin, and share 50–79% amino acid sequence identity. This review focuses on structural studies of only these four enzymes, including their orthologs in other *Plasmodium* spp.. Almost all original crystallographic studies were performed with PMII, but more recent work on PMIV, PMI, and HAP resulted in a more complete picture of the structure–function relationship of vacuolar PMs. Many structures of inhibitor complexes of vacuolar plasmepsins, as well as their zymogens, have been reported in the last 15 years. Information gained by such studies will be helpful for the development of better inhibitors that could become a new class of potent antimalarial drugs. This article is part of a Special Issue entitled: Proteolysis 50 years after the discovery of lysosome.

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1. Introduction

Malaria is the most prevalent human disease caused by infection by a parasite. It is estimated that 300–500 million people become ill every year, and 1–3 million of them die, mostly pregnant women and children [1,2]. The causative agents of malaria are various species of *Plasmodium*, with *P. falciparum, P. vivax, P. ovale,* and *P. malariae* being principally responsible for malaria in humans. The deadliest form of malaria is caused by *P. falciparum*. In recent years, some human cases of malaria have also been reported to result from infection by *P. knowlesi*, a parasite that infects monkeys in certain forested areas of Southeast Asia [3]. The parasites spread to people through the bites of female *Anopheles* mosquitoes. Several drugs are available for treating malaria [4], with sulfadoxine–pyrimethamine and artemisinin-based combinations [5] most commonly used in current medical practice. However, recent reports show that the number of deaths of malaria patients has increased because of development of drug resistance of *P. falciparum* and *P. vivax* [4]; multidrug-resistant strains of *P. falciparum* are now emerging in several parts of the world. Because of the rapid development of resistance to the current antimalarial drugs, discovery of their new, potent, and long-lasting replacements has become essential.

During its erythrocytic growth phase, the parasite degrades most of the host cell hemoglobin [4,6,7] and utilizes the amino acids obtained through this mechanism for biosynthesis of its own proteins [8], also reducing the colloid–osmotic pressure within the host cell to prevent its premature lysis [9]. The degradation process that takes place in the food vacuole of the parasite [6] involves a number of plasmepsins (PMs), enzymes belonging to the pepsin family of aspartic proteases [2,10]. These enzymes were initially called hemoglobinases [11], but the current name has been in common use since 1994 [12]. The total number of plasmepsins varies between different *Plasmodium* strains, with 10 PMs identified in the genome of *P. falciparum* [10]. Only four of them, PMI, PII, PMIV and histo-aspartic protease (HAP), reside in the acidic food vacuole and are presumed to be involved in hemoglobin degradation [2], whereas the other plasmepsins most likely play different roles [13,14]. In this review, the name "plasmepsin" will refer to only the vacuolar enzymes, unless specifically stated otherwise. Vacuolar PMs are highly homologous, sharing 50–79% amino acid sequence identity [15]. Due to their important role in providing nutrients for the rapidly growing parasites, these enzymes have been identified as promising targets for the development of novel antimalarial drugs [4]. Indeed, inhibitors of aspartic proteases have been shown to exhibit potent antiparasitic activity [11,16–19]. Nevertheless, it is still controversial whether inhibition of vacuolar plasmepsins is responsible

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for the biological effects of such inhibitors, since knockout studies showed that these four plasmepsins have overlapping roles in hemoglobin degradation [7]. Additionally, it has been shown that even deletion of all vacuolar PMs does not fully remove the sensitivity of the parasites to inhibitors of pepsin-like enzymes [20]. Some of these questions might only be answered if more structural and biological data for different PMs would become available.

As mentioned above, plasmepsins are pepsin-like aspartic proteases [21–24]. A molecule of a typical pepsin-like aspartic protease usually consists of a single polypeptide chain folded into two structurally similar domains. The active site is located in the cleft formed by these two domains [21], with each domain contributing a single catalytic aspartic acid residue (Asp32 and Asp215; pepsin numbering will be used consistently throughout this review) [25]. The side chains of the two aspartates and a water molecule found in the apoenzymes in their vicinity are generally coplanar and their inner carboxyl oxygens are located within hydrogen bond distance from each other. Another characteristic structural feature of this family of aspartic proteases is the presence in the N-terminal domain of a β-hairpin loop, known as "flap" [21,22]. The flap covers the active site [22] and plays an important role during catalysis. A variety of biochemical and structural studies have been done in order to elucidate the catalytic mechanism of these enzymes [22]. Although some details of the mechanism are still debatable, it is generally agreed that one aspartic acid acts as a catalytic base and the other one as a catalytic acid, activating the water molecule located between the aspartates [21,22,25,26]. It is likely that Asp215 is responsible for the initial activation of the water molecule, generating the nucleophile which attacks the amide carbon of the substrate. The tetrahedral intermediate thus generated accepts a proton from Asp32 and forms the products [21,27]. PMI, PII, and PMIV all contain these two catalytic aspartic acid residues and utilize the same catalytic mechanism [28–30]. In contrast, Asp32 is replaced by His32 in HAP, indicating that the catalytic mechanism of this enzyme must differ [2,15,31,32], but its details are not yet clarified. Recently solved crystal structures of HAP [15] disproved the previously proposed hypothesis that HAP is serine protease with aspartic protease fold [32], whereas they neither confirmed nor disproved the results of computational studies that postulated Asp215 to be the sole residue directly involved in catalytic mechanism of HAP, with His32 playing only a supporting role [31].

PMII has been the subject of by far the largest number of structural studies, most likely because it was the easiest one to crystallize (Table 1). Several structures of PMIV have also been reported in the last decade, whereas structures of PMI and HAP became available only relatively recently. Taken together, the available structural data provide insights into similarities and differences found among vacuolar plasmepsins and may provide guidance for creation of potent new inhibitors of this family of enzymes.

2. Primary structure of plasmepsins

Although four vacuolar plasmepsins (PMI, PMII, HAP, and PMIV) have been identified in *P. falciparum*, other infectious strains of the parasite contain only a single plasmepsin in their food vacuole, an ortholog of PMIV [33]. Similarly to many other proteases, plasmepsins are synthesized as inactive zymogens (proplasmepsins), which contain N-terminal prosegments that are removed during maturation [34]. The zymogen forms of PMI, PMII, HAP, and PMIV contain 452, 453, 451, and 449 amino acid residues, respectively (Fig. 1) [33,35–38]. The prosegments are generally longer in vacuolar proplasmepsins than in other eukaryotic aspartic proteases, in which they are only up to ~50 amino acids long [21,34]. By contrast, prosegments of PMI, PMII, HAP, and PMIV consist of 123, 124, 123, and 121 amino acid residues, respectively. These four plasmepsins exhibit ~63% sequence identity but are only ~35% homologous to mammalian enzymes renin and cathepsin D [36]. Sequence similarity among pepsin-like proteases does not extend to their prosegments (Fig. 1) [35], which are homologous within subfamilies such as vacuolar plasmepsins, but not throughout the whole family. Prosegments of vacuolar plasmepsins contain 21 amino acids (39p–59p) which form a transmembrane helix [35] characteristic of type II membrane proteins; this helix provides an anchor to the membrane [36]. Prosegments of other plasmepsins (*V–X*) differ in their lengths and primary structures.

Table 1
Crystal structures of vacuolar plasmepsins (*P. falciparum* unless noted otherwise) that are have been deposited in the Protein Data Bank by March 2011.

Protein	PDB code	Resol (Å)	Ligand	Year deposited	Reference	Remarks
PMI	3QRV	2.4	–	2011	[44]	
	3QS1	3.1	KNI-10006	2011	[44]	
PMII	1SME	2.7	Pepstatin A	1997	[39]	
	1PFZ	1.85	–	1999	[35]	zymogen
	1LF2	1.8	RS370	2002	[28]	
	1LF3	2.7	EH58	2002	[29]	
	1LF4	1.9	–	2002	[29]	
	1LEE	1.9	RS367	2002	[28]	
	1M43	2.4	Pepstatin A	2002	–	
	1ME6	2.7	Statine-based inhibitor ($C_{25}H_{47}N_3O_6$)	2004	[41]	
	1XDH	1.7	Pepstatin A	2005	[80]	
	1XE5	2.4	Pepstatin analog ($C_{29}H_{47}F_6N_5O_9$)	2005	[80]	
	1XE6	2.8	Pepstatin analog ($C_{28}H_{45}F_6N_5O_9$)	2005	[80]	
	2BJU	1.56	Achiral inhibitor ($C_{37}H_{44}N_4O_3$)	2005	[51]	
	2IGX	1.70	Achiral inhibitor ($C_{41}H_{49}N_5O_2$)	2006	[78]	
	2IGY	2.60	Achiral inhibitor ($C_{35}H_{48}N_4O$)	2006	[78]	
	1W6H	2.2	Inhibitor ($C_{32}H_{45}BrN_6O_7$), bulky P1 side chain	2006	[81]	
	1W6I	2.7	Pepstatin A	2006	[81]	
	2R9B	2.8	Reduced peptide inhibitor	2007	[58]	
	3F9Q	1.9	–	2009	[64]	Re-refinement of 1LF4
HAP	3FNS	2.5	–	2009	[15]	
	3FNT	3.3	Pepstatin A	2009	[15]	
	3FNU	3.0	KNI-10006	2009	[15]	
	3QVI	2.2	KNI-10395	2011	–	
	3QVC	1.9	–	2011	–	zymogen
PMIV	1QS8	2.5	Pepstatin A	1999	[40]	(*P. vivax*)
	1MIQ	2.5	–	2002	[40]	zymogen (*P. vivax*)
	1LS5	2.8	Pepstatin A	2003	[29]	
	2ANL	3.3	KNI-764	2006	[38]	*P. malariae*