Activity and inhibition of plasmepsin IV, a new aspartic proteinase from the malaria parasite, *Plasmodium falciparum*

David M. Wyatt, Colin Berry*

Cardiff School of Biosciences, Cardiff University, P.O. Box 911, Cardiff CF1 3US, Wales, UK

Abstract A new aspartic proteinase from the human malaria parasite *Plasmodium falciparum* is able to hydrolyse human haemoglobin at a site known to be the essential primary cleavage site in the haemoglobin degradation pathway. Thus, plasmepsin IV may play a crucial role in this critical process which yields nutrients for parasite growth. Furthermore, synthetic inhibitors known to inhibit parasite growth in red cells in culture are able to inhibit the activity of this enzyme in vitro. As a result, plasmepsin IV appears to be a potential target for the development of new antiparasitic drugs. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Malaria; Aspartic proteinase; New plasmepsin; *Plasmodium*

1. Introduction

In its intra-erythrocytic stages, the human malaria parasite, *Plasmodium falciparum*, degrades up to 80% of the haemoglobin in the infected red blood cell in order to provide nutrients for parasite growth and development [1,2]. This process, which occurs within the parasite digestive vacuole, is mediated by aspartic, cysteine and metallo proteinases, and blockade by inhibitors of these enzymes results in parasite death [3,4]. Current evidence suggests that aspartic proteinases may be responsible for initiating this haemoglobin degradation pathway [5] and two such enzymes, plasmepsins I and II, have been identified within the digestive vacuole [2] and characterised [6]. Crystal structures have been solved for plasmepsin II in its mature enzyme form [7] and a truncated form of the zymogen, proplasmepsin II [8]. We have recently reported [9] that as many as nine aspartic proteinases can be identified within the sequences that have accumulated in the databases as a result of the *P. falciparum* genome project. Full-length cDNA sequences of two of the putative new enzymes (proplasmepsins IV and VI) were detailed previously [10] and it appears that plasmepsin IV is also present within the digestive vacuole of the *P. falciparum* parasite (Roland Cooper, personal communication). The mature enzyme, plasmepsin IV, shares approximately 70% amino acid identity [10] with the previously described plasmepsins I and II. In the present report, we describe the production of proplasmepsin IV in recombinant form and detail for the first time the interaction of the new mature enzyme, plasmepsin IV, with substrates and inhibitors.

2. Materials and methods

The gene encoding a truncated form of proplasmepsin IV (beginning at residue Thr74 of this precursor [10]) was amplified from *P. falciparum* strain IT04 DNA by the polymerase chain reaction (PCR) using Advantaq DNA polymerase (Clontech, Basingstoke, UK) and forward (*GGATCCC*ACAAAACACACAACTATAGG) and reverse (*GGATCC*TTATAAATTTTTAGCTAGCTACTGCAAAAC) primers which contained *Bam*HI sites (in italics) at their respective 5′-ends. The conditions used were: 95°C for 5 min followed by 20 cycles of: 95°C for 1 min; 60°C for 1 min; 72°C for 1 min; prior to a final elongation step of 72°C for 5 min. The resultant 1145 bp product was ligated into the pGEM-T vector (Promega, Southampton, UK) and sequenced using an Applied Biosystems ABI 3100 capillary DNA sequencer. The DNA insert was excised with *Bam*HI, cloned into the expression vector pET3b and a clone with the DNA in the correct orientation and reading frame was selected after sequencing. The pET3b construct was transformed into *Escherichia coli* strain BL21DE3pLysS and induction, purification and refolding of the resultant insoluble recombinant proplasmepsin IV were performed as described previously for proplasmepsin II [11]. Autoactivation of proplasmepsin IV was accomplished by the addition of one tenth volume of 1 M sodium acetate buffer pH 4.7 (the ionic strength of which had been adjusted to 1 M by the addition of NaCl), followed by incubation at 37°C for 30 min. The resultant protein was resolved by SDS–PAGE and blotted onto polyvinylidene difluoride membrane. N-terminal sequencing by automated Edman degradation was performed by Alta Bioscience (Birmingham, UK).

The hydrolysis of chromogenic peptide substrates at pH 4.7 was monitored spectrophotometrically as described previously [4,6]. The derived kinetic constants K_m and k_{cat} always had estimated errors of < 15%. The concentration of active enzyme used in each assay was determined by active site titration with isovaleryl pepstatin as described previously [12]. K_i values for inhibitor interactions with plasmepsins IV and II were determined at pH 4.7 as described previously [12] using the chromogenic substrates Lys-Pro-Ile-Glu-Phe*Nph-Arg-Leu and Lys-Glu-Phe-Ala-Phe*Nph-Ala-Leu-Lys respectively (where * is the scissile peptide bond).

Human haemoglobin (Sigma, Poole, UK) was purified by size exclusion chromatography on Sephadex G-50 resin (Pharmacia, Uppsala, Sweden) to remove contaminating peptides. Fractions were collected and analysed for purity by SDS–PAGE. Purified haemoglobin (12.5 μg) was incubated with 250 ng pre-activated plasmepsin IV at 37°C, pH 4.7 in a final digest volume of 100 μl 0.1 M sodium acetate buffer (0.1 M ionic strength) for varying times. Digestion was stopped by the addition of SDS loading dye and boiling for 5 min prior to analysis by SDS–PAGE on a 20% polyacrylamide gel.

Erythrocyte membrane extracts were prepared as follows. Fresh human blood (20 ml, harvested into 1 mM EDTA as an anticoagulant) was centrifuged at $3000 \times g$ and the plasma, platelet and leukocyte layers were removed by aspiration. The remaining packed cells

*Corresponding author. Fax: (44)-29-20874486.
E-mail address: berry@cf.ac.uk (C. Berry).

Abbreviations: Nph, *para*-nitrophenylalanine, three letter amino acid code; Z, *para*-nitrophenylalanine, single letter amino acid code

were lysed in five volumes of lysis solution (5 mM Na_2PO_4, 5 mM $MgCl_2$) and centrifuged at $14\,000 \times g$ for 15 min. The resultant pellet (containing mostly membranous material) was washed a further four times in five volumes of buffer until no further haemolysis was evident. The pelleted membrane fraction was washed a further three times in two volumes of 0.1% (w/v) saponin (Sigma, Poole, UK) by centrifugation ($14\,000 \times g$) and resuspending prior to final resuspension in 3 ml of the same solution. Digests were performed as follows: 17 μl of membrane extract was incubated with 10 μl (0.5 μg) of either plasmepsin II or plasmepsin IV, over a range of pH values. The pH was maintained by the addition of 10% (v/v) of the appropriate buffer, (1 M sodium acetate pH 4.7; 1 M MES pH 5.5; or 1 M MOPS pH 6.6) in a final reaction volume of 1 ml. Samples were incubated for 16 h at 37°C and the final pH value was confirmed at the end of each digestion period using a standard laboratory pH probe. Digests were individually concentrated in Microspin Concentrators (Amicon Inc., Beverley, MA, USA) to a final volume of 20 μl, whereupon 5 μl of SDS loading buffer was added prior to analysis by SDS–PAGE on a 10.5% gel.

3. Results and discussion

The sequence of the proplasmepsin IV DNA amplified by PCR as described in Section 2 was identical to that first reported by Humphreys et al. [10] with the exception of an AAA–AGA substitution in the codon for Lys179→Arg. The arginine codon was detected in three independent amplification reactions so that there would appear to be a natural polymorphism in the basic residue encoded at position 179.

The DNA encoding the Arg179 variant was expressed in *E. coli* as described in Section 2 and the resultant recombinant proplasmepsin IV was solubilised and refolded using the same protocols that were elucidated initially for recombinant proplasmepsin II [11]. To confirm the identity of the 44 kDa proplasmepsin IV, N-terminal sequencing was performed and gave the sequence XXXTGGQQMGRDP (where the identity of amino acid X could not be determined). The pET3b vector encodes the sequence MASMTGGQQM-GRDP, so that the recombinant protein was coincident at its N-terminus with that of this N-terminal leader peptide provided by the vector (but lacking the initiator methionine). Incubation of the 44 kDa proplasmepsin IV at pH 4.7 resulted in its conversion to a protein which migrated on SDS–PAGE with an apparent molecular weight of 38 kDa (data not shown). This conversion was inhibited by inclusion of pepstatin. N-terminal analysis of the 38 kDa band gave two overlapping sequences FKSGYA and KSGYAQ, indicating that autoactivation had taken place at two adjacent residues (marked by ↓) in the following sequence:

- Proplasmepsin IV ∼ K E S F K F ↓ F ↓ K S G Y A Q ∼
- Proplasmepsin I   ∼ K E S L K F   F   K T G L T Q ∼
- Proplasmepsin II   ∼ K E S V N F ↑ L   N S G L T K ∼

Thus, the autoactivation appears to have taken place at a comparable site in the propart of proplasmepsin IV to that previously observed for proplasmepsin II [6], as indicated above. However, the sequence of the propart of proplasmepsin IV in this region is closely similar to that of proplasmepsin I (see above). Despite extensive efforts previously reported [4], recombinant proplasmepsin I was found to be incapable of undergoing autoactivation. Since plasmepsin IV clearly can cleave within the ∼ Ser-(Phe/Leu)-Lys-Phe-Phe-Lys-(Ser/Thr)-Gly ∼ sequence, it may be possible that plasmepsin IV initiates cleavage of the proplasmepsin I precursor. Proteomic analysis of the digestive vacuole (Roland Cooper, personal communication) has indicated that plasmepsin IV is a constit-
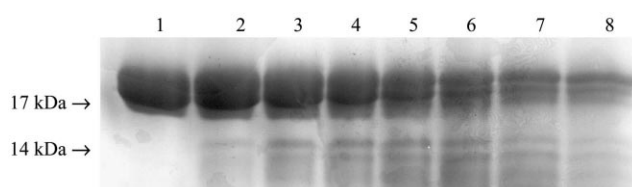

Fig. 1. Degradation of haemoglobin by plasmepsin IV. Human haemoglobin (12.5 μg) was incubated with recombinant plasmepsin IV (250 ng) at 37°C in 0.1 M sodium acetate buffer pH 4.7 for the following times: lane 1, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min; lane 5, 60 min; lane 6, 120 min; lane 7, 240 min; lane 8, 480 min. Incubation of substrate alone for 480 min resulted in no change in the protein profile of the sample (not shown).

uent of the parasite digestive vacuole, along with plasmepsin I (and plasmepsin II [2]).

The activity of recombinant plasmepsin IV against native human haemoglobin was investigated. At pH 4.7, the enzyme was able to degrade haemoglobin so that no bands remained visible on SDS–PAGE (results not shown). When an excess of haemoglobin was used as substrate in a 5 min incubation, a major early intermediate in the degradation was observed at approximately 14 kDa (Fig. 1). N-terminal analysis of this band revealed the sequence Leu-Ser-Phe-Pro-Thr consistent with a cleavage of the α-globin chain at the Phe33–Leu34 bond in the hinge region of this molecule. This is precisely the bond believed to be the primary cut site necessary to initiate haemoglobin degradation in the parasite digestive vacuole and is the only cut site which plasmepsins I and II share in common when human haemoglobin is used as substrate [2]. Thus, it appears that, in common with the other digestive vacuole plasmepsins, plasmepsin IV may also have the potential to initiate the vacuolar haemoglobin digestion pathway.

In addition to haemoglobin, the spectrin associated with the membrane of the host red blood cell is also depleted during infection by *Plasmodium* parasites [13]. Membrane extracts prepared from human erythrocytes as described in Section 2 displayed two prominent bands on SDS–PAGE that correspond to the α- (260 kDa) and β- (225 kDa) chains of spectrin (Fig. 2, lane 1). Incubation of the membrane extract with the recombinant plasmepsin IV at pH 4.7 resulted in essentially complete degradation of all visible bands on SDS–PAGE
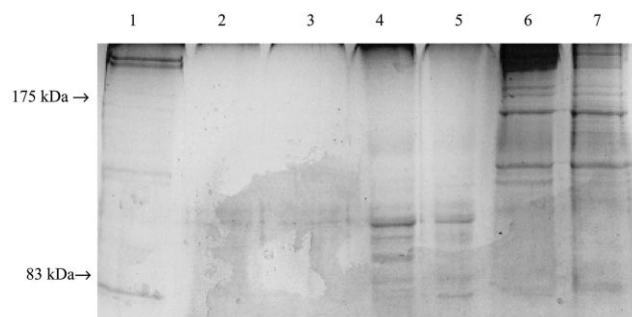

Fig. 2. Digestion of erythrocyte membrane extract by recombinant plasmepsins IV and II. Lane 1: erythrocyte membrane extract alone; lanes 2, 4 and 6: membrane extract incubated with recombinant plasmepsin IV at pH 4.7, 5.5 and 6.6 respectively; lanes 3, 5 and 7: membrane extract incubated with recombinant plasmepsin II at pH 4.7, 5.5 and 6.6 respectively. Incubation of substrate alone for 480 min resulted in no change in the protein profile of the sample (not shown).

Table 1
Kinetic parameters for the hydrolysis of chromogenic peptide substrates

Peptide sequence	Plasmepsin IV			Plasmepsin I			Plasmepsin II		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
KEFAF*ZALK	35	13	370	45[b]	3[b]	70[b]	40[b]	17[b]	430[b]
KEFNF*ZALK	20	20	1000	20[a]	2[a]	100[a]	25[a]	7[a]	280[a]
KERVF*ZALK	11	6	550	20[b]	0.3[b]	20[b]	30[b]	7[b]	230[b]
KPIEF*ZRL	36	5	140	–	< 0.01[b]	–	20[b]	1[b]	50[b]

Values were measured at 37°C, pH 4.7, ionic strength = 0.1 M. In the peptide sequences, * represents the cleavage point. Values for recombinant plasmepsins I and II are taken from [a]Tyas et al. [6] and [b]Tyas [16].

(Fig. 2, lane 2). At pH 5.5, the extent of digestion was less (Fig. 2, lane 4) even after an incubation period of 16 h. At pH 6.6, although proteolysis to generate intermediates of smaller size was still occurring (Fig. 2, lane 6), the intermediates were, in general, larger in turn than those that were detected at pH 5.5. Indeed, the pattern of spectrin digestion by plasmepsin II at pH 6.6 (Fig. 2, lane 7) was comparable to that observed with plasmepsin IV (lane 6). It has been suggested previously that spectrin degradation in infected red blood cells does not occur in the digestive vacuole but rather is accomplished at a higher pH within the cytosol of the red cell and it was postulated that this proteolysis might be accomplished by plasmepsin II which has been detected in the host cell cytosol [14]. Recent evidence suggesting local regions of acidification within parasitised red cells may also prove consistent with plasmepsin II activity against spectrin [15] and, since plasmepsin IV is also capable of spectrin degradation, it would appear that it may also be a candidate in this process although its existence outside the digestive vacuole is yet to be established.

The ability of recombinant plasmepsin IV to cleave several synthetic peptide substrates was also examined. The kinetic parameters obtained with each of four substrates (Table 1) were similar (within a factor of 3–4-fold) to their counterpart values reported previously for cleavage of each of these substrates by recombinant plasmepsin II [6]. In this regard then, the active sites of the two enzymes would appear to be comparable. Direct comparisons with plasmepsin I are more complicated because of the difficulties in obtaining this enzyme in an active recombinant form. Plasmepsin IV was potently inhibited by the acetyl, isovaleryl and isobutyryl variants of pepstatin (Table 2). The shorter lactoyl-pepstatin, which has a hydrophilic lactoyl substituent in P3 in place of the Val residue of the other three variants of pepstatin, was a considerably poorer inhibitor not only of plasmepsin IV but also of plasmepsin II. In contrast, three chemically synthesised inhibitors which we have tested previously [4] as inhibitors of plasmepsin I and plasmepsin II (and the structures of which are

detailed in Moon et al. [4]) were found to be 16–80 times more potent inhibitors of plasmepsin IV than plasmepsin II. These compounds thus permit discrimination between the active sites of plasmepsin IV and plasmepsin II where the synthetic peptide substrates do not. The potencies of Ro40-4388 and Ro40-5576 towards plasmepsin IV (Table 2) are closely similar to those reported previously [6] for plasmepsin I. Plasmepsin IV is the most sensitive of the digestive vacuole enzymes to Ro42-1118, which is 6-fold and 80-fold more potent against plasmepsin IV than plasmepsins I and II respectively. Since this compound is also an inhibitor of *P. falciparum* growth in red blood cells in culture [4], this may suggest plasmepsin IV as a possible target for the action of this compound in the parasite.

From the above analyses, it is clear that plasmepsin IV has an active site that is similar to, but yet distinct from those of plasmepsin I and plasmepsin II so that the three enzymes might well act in concert in the digestive vacuole of *P. falciparum* feeding within the confines of the red blood cell. While this might suggest that inhibition of individual enzymes might be insufficient to kill the parasite, the broad similarities between the digestive vacuole plasmepsins may permit the development of compounds able to inhibit all three enzymes to produce a drug capable of imposing a multiple blockade on the pathway of haemoglobin digestion.

References

[1] Francis, S.E., Gluzman, I.Y., Oksman, A., Knickerbocker, A., Mueller, R., Bryant, M.L., Sherman, D.R., Russel, D.G. and Goldberg, D.E. (1994) EMBO J. 13, 306–317.
[2] Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L. and Goldberg, D.E. (1994) J. Clin. Invest. 93, 1602–1608.
[3] Rosenthal, P.J., McKerrow, J.H., Aikawa, M., Nagasawa, H. and Leech, J.H. (1988) J. Clin. Invest. 82, 1560–1566.

Table 2
Inhibition of plasmepsin IV

Inhibitor	Plasmepsin IV K_i (nM)	Plasmepsin I K_i (nM)	Plasmepsin II K_i (nM)
Ac-Val-Val-Sta-Ala-Sta	0.2	nd	0.6
Iva-Val-Val-Sta-Ala-Sta	0.1	0.7[a]	< 0.1
Ibu-Val-Val-Sta-Ala-Sta	< 0.1	nd	0.1
Lac-Val-Sta-Ala-Sta	25	nd	100
Ro40-4388	10	4[a]	700[a]
Ro40-5576	15	6[a]	250[a]
Ro42-1118	50	300[b]	4000[a]

Values were measured at pH 4.7 as described in the legend to Fig. 1. nd = not determined. The interaction of isobutyryl pepstatin with plasmepsin IV was so tight that problems of mutual depletion were encountered, so that an accurate K_i value could not be measured using the methodology employed. Values for recombinant plasmepsins I and II with synthetic inhibitors are derived from [a]Tyas et al. [6] and [b]Tyas [16].

[4] Moon, R.P., Tyas, L., Certa, U., Rupp, K., Bur, D., Jaquet, C., Matile, H., Loetscher, H.-R., Grueninger-Leitch, F., Kay, J., Dunn, B.M., Berry, C. and Ridley, R.G. (1997) Eur. J. Biochem. 244, 552–560.

[5] Goldberg, D.E., Slater, A.F.G., Beavis, R., Chait, B., Cerami, A. and Henderson, G.B. (1991) J. Exp. Med. 173, 961–969.

[6] Tyas, L., Gluzman, I., Moon, R.P., Rupp, K., Westling, J., Ridley, R.G., Kay, J., Goldberg, D.E. and Berry, C. (1999) FEBS Lett. 454, 210–214.

[7] Silva, A.M., Lee, A.Y., Gulnik, S.V., Majer, P., Collins, J., Bhat, T.N., Collins, P.J., Cachau, R.E., Luker, K.E., Gluzman, I.Y., Francis, S.E., Oksman, A., Goldberg, D.E. and Erickson, J.W. (1996) Proc. Natl. Acad. Sci. USA 93, 10034–10039.

[8] Bernstein, N.K., Cherney, M.M., Loetscher, H., Ridley, R.G. and James, M.N.G. (1999) Nat. Struct. Biol. 6, 32–37.

[9] Coombs, G.H., Goldberg, D.E., Klemba, M., Berry, C., Kay, J. and Mottram, J.C. (2001) Trends Parasitol. 17, 532–537.

[10] Humphreys, M.J., Moon, R.P., Klinder, A., Fowler, S.D., Rupp, K., Bur, D., Ridley, R.G. and Berry, C. (1999) FEBS Lett. 463, 43–48.

[11] Hill, J., Tyas, L., Phylip, L.H., Kay, J., Dunn, B.M. and Berry, C. (1994) FEBS Lett. 352, 155–158.

[12] Jupp, R.A., Dunn, B.M., Jacobs, J.W., Vlasuk, G., Arcuri, K.E., Veber, D.F., Perlow, D.S., Payne, L.S., Boger, J., De Laszlo, S., Chakravarty, P.K., Ten Broeke, J., Hangauer, D.G., Ondeyka, D., Greenlee, W.J. and Kay, J. (1990) Biochem. J. 265, 871–878.

[13] Weidekamm, E., Wallach, D., Lin, P. and Hendricks, J. (1973) Biochim. Biophys. Acta 323, 539–546.

[14] Le Bonniec, S., Deregnaucourt, C., Redeker, V., Banerjee, R., Grellier, P., Goldberg, D.E. and Schrével, J. (1999) J. Biol. Chem. 274, 14218–14223.

[15] Hayashi, M., Yamada, H., Mitamura, T., Horii, T., Yamamoto, A. and Moriyama, Y. (2000) J. Biol. Chem. 275, 34353–34358.

[16] Tyas, L. (1997) Plasmepsins I and II, malarial parasite aspartic proteinases, Ph.D. Thesis, Cardiff University, Cardiff.