Molecular & Biochemical Parasitology 197 (2014) 56–63

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

The zymogen of plasmepsin V from *Plasmodium falciparum* is enzymatically active

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ARTICLE INFO

Article history:
Received 26 February 2014
Received in revised form 15 October 2014
Accepted 16 October 2014
Available online 25 October 2014

Keywords:
Plasmepsin
Zymogen
Refolding
Prosegment
Protease inhibitor
Plasmodium falciparum

ABSTRACT

Plasmepsin V, a membrane-bound aspartic protease present in *Plasmodium falciparum*, is involved in the export of malaria parasite effector proteins into host erythrocytes and therefore is a potential target for antimalarial drug development. The present study reports the bacterial recombinant expression and initial characterization of zymogenic and mature plasmepsin V. A 484-residue truncated form of proplasmepsin (Glu37–Asn521) was fused to a fragment of thioredoxin and expressed as inclusion bodies. Refolding conditions were optimized and zymogen was processed into a mature form *via* cleavage at the Asn80-Ala81 peptide bond. Mature plasmepsin V exhibited a pH optimum of 5.5–7.0 with K_m and k_{cat} of 4.6 μM and 0.24 s^{-1}, respectively, at pH 6.0 using the substrate DABCYL-LNKRLLHETQ-E(EDANS). Furthermore, the prosegment of proplasmepsin V was shown to be nonessential for refolding and inhibition. Unexpectedly, unprocessed proplasmepsin V was enzymatically active with slightly reduced substrate affinity (~2-fold), and similar pH optimum as well as turnover compared to the mature form. Both zymogenic and mature plasmepsin V were partially inhibited by pepstatin A as well as several KNI aspartic protease inhibitors while certain metals strongly inhibited activity. Overall, the present study provides the first report on the nonessentiality of the prosegment for plasmepsin V folding and activity, and therefore, subsequent characterization of its structure-function relationships of both zymogen and mature forms in the development of novel inhibitors with potential antimalarial activities is warranted.

1. Introduction

Malaria afflicts up to 500 million people annually of which 1–2 million cases are deadly [1]. It is primarily spread through the bite of *Anopheles* mosquitos which transmit protozoans of the genus *Plasmodium*, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [2]. *P. falciparum*, the most virulent malaria parasite, is responsible for nearly all malarial deaths [3]. After nearly a century of success in reducing its spread there has been a resurgence of malaria during the past two decades due to increased resistance of the parasites to available drugs and insecticides. Hence, there is an urgent need for engineering new antimalarial compounds having novel mechanisms of action.

Ten plasmepsins (PMs) have been reported from the genome of *P. falciparum*, four of which (PMs I, II, IV and histo-aspartic protease) have been shown to be involved in hemoglobin degradation in the food vacuole of the parasite [4–6]. These have been potential targets for developing novel antimalarials [4], however, it was recently reported that the endoplasmic reticulum aspartic protease PMV [7] could be a superior target for malaria control [8–10]. In order to survive and promote its virulence, the parasite must export hundreds of its proteins beyond an encasing vacuole and membrane into the host red blood cell [11,12]. These exported proteins are synthesized in the endoplasmic reticulum having the *Plasmodium* export element (PEXEL), a pentameric motif (R/KxLxE/Q/D) [11,12]. PMV is responsible for cleavage of PEXEL [8,9,13] and is thus required

Abbreviations: DABCYL, 4-((4-(dimethylamino)phenyl)azo)benzoic acid; EDANS, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid; KNI, kinostatin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

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http://dx.doi.org/10.1016/j.molbiopara.2014.10.004
0166-6851/

for parasite protein export mediation [8,9,13,14]. For this reason, PMV could be an important target in the development of novel, effective antimalarial drugs [8–10] particularly in consideration of recently published findings regarding the engineering of a PEXEL-mimetic inhibitor that was shown to effectively kill *P. falciparum* parasites *via* direct action against PMV *in vivo* [15], a potentially critical advance in the fight against malaria.

In order to facilitate the development of specific inhibitors with antimalarial activities, the elucidation of structure–function relationships of PMV, particularly with respect to its modes of proteolysis, inhibition and activation, are important starting points in working toward a level of understanding that facilitates structure elucidation, and ultimately inhibitor design. The present study reports the recombinant expression, pH conditions for optimal activity, inhibitor testing, and most importantly the finding that the prosegment is apparently non-essential for obtaining proteolytic activity and ligand binding.

2. Materials and methods

2.1. Materials

pET32b(+) and pET19b(+) vectors, *Escherichia coli* Rosetta-gami B (DE3)pLysS, BugBuster™ reagent and u-MAC™ cartridges were purchased from Merck KGaA (Darmstadt, Germany). A synthetic 44-residue peptide corresponding to the PMV prosegment (ENKIDNVGKKIENVGKKIGDMENKNDNVENKND-NVGNKNDNVKN) was purchased from GenicBio (Shanghai, China). A quenched fluorescent synthetic peptide substrate (HRPII; 4-(4-dimethylaminophenyl) diazenylbenzoic acid (DABCYL)-LNKRLLHETQ-E(5-[(2-Aminoethyl)amino]naphthalene-1-sulfonic acid) (EDANS), and L→A mutant HRPII DABCYL-LNKRLAHETQ-E(EDANS), was purchased from CanPeptide (Pointe-Claire, QC, Canada). All other chemicals and media were obtained from Fisher Scientific Canada (Nepean, ON, Canada) or Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cloning and construction of soluble expression vectors

The gene encoding for zymogenic PMV (proPMV) [9] was amplified from the genomic DNA of *Plasmodium falciparum* 3D7 (MR4/American Type Culture Collection, Manassas, VA, USA) using primers PMVF109 (5′GCACCATGGAAAATAAAATTGACAATGTTG) and PMVR1563 (5′AATCCATGGCTAATTAGATGGGCATTTAGATTC). Product was digested with *Nco*I and ligated into pET32b(+) yielding pET32b-proPMV wherein *proPMV* was fused to a fragment of *thioredoxin* (*trx*).

2.3. Subcloning and construction of insoluble expression vectors

E. coli codon-optimized synthetic genes for mature PMV and proPMV were purchased from GenScript (Piscataway, NJ, USA). The former was amplified using primers *optVF241* 5′GCAC-CATGGCGAGCTCTGATCTGTATAAATAC and *optVR1563* 5′AATCTCG-AGTTAATGGTGATGGTGATGGTGGTTACTCGGACACTTAGATTC, and subsequently subcloned into pET19b(+) at the *Nco*I and *Xho*I restriction sites to produce *pET19b-PMV* containing a C-terminal His_6 tag. *ProPMV* was amplified using primers *optVF109Htag* 5′GCACCATGGCTCATCATCATCATCATCATGAAAACAAGATCGATAA-CGTG and *optVR1563* 5′AATCTCGAGTTAGTTACTCGGACACTTAG-ATTC, and inserted at the *Nco* I and *Xho* I restriction sites yielding *pET19b-proPMV*. Control protein products were made as well by producing Asp118Ala mutants (Ala knockout of the critical catalytic Asp118) for both *PMV* and *proPMV*. The mutants were made using QuickChange™ Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA, USA).

2.4. Expression, solubilization, refolding, and purification

E. coli Rosetta-gami B (DE3)pLysS (for soluble expression) transformed with expression vector constructs were cultured, induced and harvested as per the manufacturer's instructions. Frozen cell pellets were resuspended in BugBuster™ cell lysis reagent and incubated at room temperature for 20 min with gentle shaking. Soluble and insoluble materials were separated by centrifugation at 16,000 × *g* for 20 min at 4 °C. Insoluble protein was solubilized in 50 mM Tris–HCl pH 9.0 buffer containing 8 M urea and 10 mM β-mercaptoethanol. To optimize refolding conditions, solubilized protein solution was diluted 20-fold in various refolding buffers having different pH, ratio of oxidized:reduced glutathione, as well as addition of varying concentrations of urea, NaCl, glycerol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), $MgSO_4$, Tween-20, Triton X-100, sucrose, arginine, 2-(cyclohexylamino)ethanesulfonic acid (CHES) and KCl. Refolding efficiency was assessed as per Russo et al. [9].

Refolded protein was purified by size-exclusion chromatography using a Superose12 10/300 GL™ column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in 50 mM Tris–HCl pH 8.0 followed by MonoQ™ HP anion exchange pre-equilibrated with 50 mM Tris–HCl pH 8.0, and eluted with a linear gradient to 1 M NaCl. Fractions were screened for target protein by non-reducing SDS–PAGE and enzyme activity assays. Fractions containing monomers with relatively high activity were pooled and further purified by cobalt affinity chromatography using a 1 mL u-MAC™ cartridge. Eluent was dialyzed against 50 mM Tris–HCl pH 8.0 containing 10 mM EDTA at 4 °C (10,000-fold dilution) and was subsequently stored at −20 °C.

2.5. Activation of PMV

In order to determine optimal activation pH, 2 μg of purified Trx-proPMV were incubated in pH 5.0–9.5 buffers at 37 °C for 12 h. Activation was determined by SDS-PAGE band-shifting.

2.6. SDS-PAGE and N-terminal sequencing

SDS-PAGE was performed according to the method of Laemmli [16] using a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). N-terminal sequence analysis was done by the Advanced Protein Technology Centre (Toronto, ON, Canada).

2.7. Proteolytic activity pH-optimum

pH-optima were determined using 10 nM enzyme, 5 μM substrate HRPII at 37 °C and pH 3.0–9.0. Assay detection was performed using a Victor2™ 1420 Multilabel Counter (Perkin Elmer, Woodbridge, ON, Canada) with $\lambda_{excitation}$ at 335 nm and $\lambda_{emission}$ at 500 nm [17].

2.8. Enzyme kinetics and inhibition

Kinetic parameters were determined at 37 °C in 50 mM MES buffer pH 6.0 containing 0.005% Tween-20 using 10 nM PMV or proPMV and 0.10–12 μM HRPII peptide substrate. Initial reaction rates were determined by converting the slope of the linear portion of the curve (Δfluorescence min^{-1}) to μmol min^{-1} using a conversion factor of 845,582 μM^{-1} derived from a standard curve for HRPII substrate fully digested by commercial yeast proteinase A (Sigma–Aldrich, Oakville, ON, Canada). Non-linear regression analysis using the Michaelis–Menten model was applied