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Characterization of the monomer–dimer equilibrium of recombinant histo-aspartic protease from *Plasmodium falciparum*

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ABSTRACT

Histo-aspartic protease (HAP) from *Plasmodium falciparum* is an intriguing aspartic protease due to its unique structure. Our previous study reported the first recombinant expression of soluble HAP, in its truncated form (lys77p-Leu328) (p denotes prosegment), as a thioredoxin (Trx) fusion protein Trx–tHAP. The present study found that the recombinant Trx–tHAP fusion protein aggregated during purification which could be prevented through the addition of 0.2% CHAPS. Trx–tHAP fusion protein was processed into a mature form of tHAP (mtHAP) by both autoactivation, and activation with either enterokinase or plasmepsin II. Using gel filtration chromatography as well as sedimentation velocity and equilibrium ultracentrifugation, it was shown that the recombinant mtHAP exists in a dynamic monomer–dimer equilibrium with an increasing dissociation constant in the presence of CHAPS. Enzymatic activity data indicated that HAP was most active as a monomer. The dominant monomeric form showed a K_m of 2.0 μM and a turnover number, k_{cat}, of 0.036 s^{-1} using the internally quenched fluorescent synthetic peptide substrate EDANS–CO–CH_2–CH_2–CO–Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro–Dap–(DABCYL)–OH (2837b) at pH 5.2.

1. Introduction

Malaria is a devastating disease that infects millions of people and kills 1–2 million people annually. In 2006, there were an estimated 247 million cases of malaria, causing nearly a million deaths, mostly children under 5 years [1]. The development of *Plasmodium falciparum* resistance to drugs such as chloroquine and sulphadoxine/pyrimethamine is a major challenge for malaria control; therefore, new treatment methods and alternative drugs are needed [2]. The plasmepsins (PMs) produced by the *Plasmodium* parasite are aspartic proteases and have been considered attractive targets for new antimalarial drugs [3].

The *P. falciparum* parasite, the most lethal of all *Plasmodium* parasites, is known to encode ten PMs (PMs I, II and IV–X and histo-aspartic protease (HAP)) [4], four of which (PM I, PM II, PM IV and HAP) reside within the food vacuole and are involved not only in human hemoglobin degradation [5], but also in other cellular pathways requiring optimal vacuolar function such as autophagy [6]. PM V is localized in the endoplasmic reticulum [7] and directs *Plasmodium* proteins for export into the host erythrocyte [8,9]. PMs I, II and IV are considered to be 'classic' aspartic proteases, i.e., peptidase family A1, retaining a catalytic dyad of two aspartic acid residues. In contrast, HAP, which is designated A01.043 in the peptidase family A1, is characterized by one of the catalytic aspartic acids, Asp34 (numbering based on the native mature form [10]), being replaced with a histidine and the substitution of the normally conserved Tyr 77 and Val 78 residues, of the flap region of HAP, with serine and lysine, respectively [11]. On the basis of the aforementioned amino acid substitutions, HAP is thought to function through an alternative catalytic mechanism as compared to other aspartic proteases [5,11–15]. Molecular modeling has led to various proposed modes of action for HAP [12,13]. Andreeva et al. suggested that HAP might act like a serine protease with a catalytic triad of Ser 37-His 34-Asp 214 and an oxyanion hole formed by Ser 38 and Asn 39 [12]. Alternatively, Bjelic and Aqvist proposed that HAP functions through the direct participation of only Asp 214 with His 34 providing critical stabilization to the reaction [13]. Experimental evidence to test these alternative hypotheses has been restricted by the inability to recombinantly express HAP. Xiao et al. reported for the first time, the recombinant expression of soluble, active HAP using a truncated form of HAP (Lys77p-Leu328) fused to thioredoxin in the pET32b(+) vector in *Escherichia coli* Rosetta-gami B (DE3)pLysS [14]. Subsequently, Parr et al. characterized three mutant HAP proteins, H34A, S37A and D214A and suggested a novel mode of catalysis with a single aspartic acid residue performing both the acid and base roles [15]. Recently, the X-ray structures of the recombinant mature HAP,

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as an apoenzyme and as complexes with two inhibitors, pepstatin A and KNI-10006, have been solved [16]. The structures, however, did not provide an unambiguous answer as to the true nature of the catalytic mechanism. The structure of HAP complexed with pepstatin A exhibited a structure similar to other pepsin-like aspartic proteases. The apoenzyme, however, formed a tight dimer with the active site containing a zinc ion tightly bound to His34 and Asp214 from one monomer to Glu278 to the other monomer, with the coordination resembling a metalloprotease. This study raised questions regarding of the oligomeric status of mtHAP in solution and whether or not mtHAP was active as a dimer. Limited studies have appeared in the literature regarding the oligomeric forms of plasmepsin and especially HAP. Asojo et al. reported several potential dimeric forms of plasmepsins based on crystallographic and non-crystallographic symmetry [17], however, it has been reported that PM II exists mainly as a monomer in solution as demonstrated by gel filtration chromatography and analytical ultracentrifugation [18]. In the only report on HAP, Banajee et al. reported that the purified native active protein migrated as a monomer in the gel filtration chromatography [5]. Therefore, in the present study, gel filtration chromatography and analytical ultracentrifugation were used to study the molecular nature of the active form of HAP. In addition, the processing of the recombinant Trx–tHAP fusion protein to mature HAP was also investigated.

2. Materials and methods

2.1. Expression and purification of the fusion Trx–tHAP and mature mtHAP

Expression of the recombinant Trx–tHAP fusion protein was similar to that described by Xiao et al. [14]. Purification of the fusion Trx–tHAP and mature mtHAP was modified as follows. Cell pellet of 1 l culture was lysed in 50 ml of 1× BugBuster™ reagent (Novagen, Madison WI, USA) containing 1 μl Benznase (Novagen) and incubated at room temperature for 40 min with gentle shaking. The sample was then centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatant was applied on a HisSelect™ Cartridge (6.4 ml) (Sigma–Aldrich, Oakville ON, Canada); the column was first washed with 50 mM sodium phosphate/0.3 M NaCl/10 mM imidazole pH 7.5 washing buffer and then with 10% elution buffer (50 mM sodium phosphate/0.3 M NaCl/250 mM imidazole pH 7.5), and finally eluted with 50% elution buffer. The eluted proteins were concentrated in 50 mM sodium phosphate (pH 7.5) containing 0.2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in an Ultracel YM50 Centricon (Millipore, Billerica, USA) and further purified by gel filtration on a Superose™ 12 10/300 GL (GE Healthcare, Uppsala, Sweden) column equilibrated with 50 mM sodium phosphate/150 mM NaCl pH 7.5 (containing 0.2% CHAPS) to obtain the fusion Trx–tHAP protein. Fractions with the fusion protein were collected and washed with 50 mM sodium phosphate buffer (pH 7.5) (containing 0.2% CHAPS). The fusion Trx–tHAP protein was then activated with enterokinase (EK) (Sigma–Aldrich) (EK: Trx–tHAP = 1:25) in 50 mM MES (pH 6.5) (containing 0.2% CHAPS) at 37 °C for 2 days. Subsequent to confirmation of successful activation via SDS-PAGE, the activated mature tHAP (mtHAP) was purified by washing the sample with 50 mM MES (pH 6.5) in an Ultracel YM30 Centricon (Millipore, Billerica, USA) to remove the cleaved prosegment and thioredoxin to mtHAP. The mtHAP was further purified by gel filtration using a Superose™ 12 10/300 GL column equilibrated with 50 mM MES/150 mM NaCl pH 6.5 (containing 0.2% CHAPS). The elution fractions with mature mtHAP were then subject to size exclusion chromatography and analytical ultracentrifugation analysis.

2.2. Size exclusion chromatography

Gel filtration was carried out on an AKTA™ FPLC system (GE Healthcare, Uppsala, Sweden) via a SuperoseTM 12 10/300 GL column equilibrated with 50 mM sodium phosphate/150 mM NaCl pH 7.5 or 50 mM MES/150 mM NaCl pH 6.5, with or without 0.2% CHAPS. The flow rate was 0.5 ml/min and the elution was monitored at 280 nm.

2.3. Analytical ultracentrifugation

Sedimentation velocity experiments were performed in the Beckman Optima XL-A analytical ultracentrifuge using an An60Ti rotor (Beckman, Fullerton, CA) at the Biomolecular Interactions & Conformations Facility at the University of Western Ontario (London, Ontario, Canada). Samples at 0.5 mg/ml, 0.33 mg/ml and 0.25 mg/ml in buffer containing 50 mM MES, pH 6.5, 150 mM NaCl, and 0.2% CHAPS were run in 2-sector cells with epon-charcoal centerpieces, at 40,000 rpm and 5 °C. Absorbance was measured at 280 nm every 10 min, using 0.003-cm radial steps and averaging over three readings. The data were fitted using a size distribution analysis provided by the program SEDFIT [19]. Observed sedimentation coefficients were corrected to $s_{20,w}$, the values expected in water at 20 °C, by the standard method.

Sedimentation equilibrium was conducted using the above ultracentrifuge in two experiments. In the first experiment, the same buffer as in the sedimentation velocity experiments was used. Various sample concentrations (0.5 mg/ml, 0.33 mg/ml and 0.25 mg/ml) were run in 6-channel cells with epon-charcoal centerpieces, at rotor speeds of 16,000 rpm, 22,000 rpm and 28,000 rpm and 5 °C. Absorbance was measured at 280 nm, using 0.002-cm radial steps and averaging over 10 readings. The extinction coefficient was calculated to be 37,350 M^{-1} cm^{-1}, based on the initial absorbance and experimentally determined protein concentration. The buffer density was measured with a 25-ml specific gravity bottle to be 1.013 g/ml. The partial specific volume of mtHAP was calculated from the inferred amino acid composition to be 0.733 ml/g, using the freeware program SEDNTERP [produced by Tom Laue of the University of New Hampshire]. Data were analyzed using models constructed in Prism 4 (Graphpad) as described previously [20]. The second experiment was done in buffer containing 50 mM MES, pH 6.5, 150 mM NaCl and CHAPS at 0%, 0.1%, 0.2%, or 0.3%. The initial protein concentration was 0.33 mg/ml. The temperature, rotor speeds and data collection were as described above.

2.4. SDS-PAGE and N-terminal sequence of the active recombinant HAP

SDS-PAGE was performed according to the method of Laemmli (1970) [21] in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). Protein samples were mixed with sample buffer in the presence of β-mercaptoethanol and SDS, heated for 5 min at 100 °C, and loaded onto a 15% polyacrylamide gel with SDS. Gels were run at a constant voltage (200 V) for 1 h. The gels were developed with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA).

Mature tHAP protein activated by PM II was subjected to SDS-PAGE and electroblotted to a polyvinylidene difluoride (PVDF) membrane for N-terminal sequence analysis by the Advanced Protein Technology Center at the Hospital for Sick Children (Toronto, ON, Canada).

2.5. Protein concentration determinations

Enzyme and protein concentrations were determined in triplicate by the DC protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard [22].