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Characterization of a new phosphatase from *Plasmodium*☆

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ABSTRACT

Plasmodium falciparum malaria is the most important parasitic disease worldwide, responsible for an estimated 1 million deaths annually. Two *P. falciparum* genes code for putative phosphoglycerate mutases (PGMases), a widespread protein group characterized by the involvement of histidine residues in their catalytic mechanism. PGMases are responsible for the interconversion between 2 and 3-phosphoglycerate, an intermediate step in the glycolysis pathway. We have determined the crystal structures of one of the *P. falciparum*'s PGMases (*Pf*PGM2) and a functionally distinct phosphoglycerate mutase from *Cryptosporidium parvum*, a related apicomplexan parasite. We performed sequence and structural comparisons between the two structures, another *P. falciparum* enzyme (*Pf*PGM1) and several other PGM family members from other organisms. The comparisons revealed a distinct conformation of the catalytically active residues not seen in previously determined phosphoglycerate mutase structures. Furthermore, characterization of their enzymatic activities revealed contrasting behaviors between the *Pf*PGM2 and the classical cofactor-dependent PGMase from *C. parvum*, clearly establishing *Pf*PGM2 as a phosphatase with a residual level of mutase activity. Further support for this function attribution was provided by our structural comparison with previously characterized PGM family members. Genetic characterization of PGM2 in the rodent parasite *Plasmodium berghei* indicated that the protein might be essential to blood stage asexual growth, and a GFP tagged allele is expressed in both blood and zygote ookinete development and located in the cytoplasm. The *P. falciparum* PGM2 is either an enzyme implicated in the phosphate metabolism of the parasite or a regulator of its life cycle.

1. Introduction

The complete genome of *Plasmodium falciparum* was sequenced in 2002 with nearly 40% of the parasite genome annotated based on sequence analysis [1]. Among the annotated genes, there are at least 6 predicted to encode proteins with a phosphoglycerate mutase domain, namely PF11_0208, PFD0660w, PF14_0094, PFC0430w, PFB0380c and PF14_0282, but only the first two were annotated as putative phosphoglycerate mutases [2]. Phosphoglycerate mutases (EC 5.4.2.1; PGMase) catalyze the reversible conversion of 2-phosphoglycerate (2-PG) to 3-phosphoglycerate (3-PG) [3]. This is an essential component of the glycolysis pathway providing 2-PG to the enzyme enolase and also of the gluconeogenesis pathway where it supplies 3-PG to the phosphoglycerate kinase.

There are two unrelated forms of the PGMase, the cofactor-dependent (dPGM) that carries out the reaction via its cofactor 2,3-bisphosphoglycerate (2,3-BPG) and the cofactor-independent form (iPGM) which is fully dependent on a divalent metal [3]. The

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cofactor-dependent dPGM is the founding member of the phosphoglycerate mutase protein family (PGM) [4]. Members of this family share a common catalytic α/β fold, designated as PGM domain, which contains a histidine residue acting as a phosphor acceptor during catalysis [5]. Phosphoglycerate mutases also share several structural features with histidine acid phosphatases. Therefore, the two families have been integrated into the histidine phosphatase superfamily [4]. Members of the PGM family function as phosphotransferases or phosphohydrolases acting upon a variety of substrates [4]. For example, the bisphosphoglycerate mutase that catalyzes the synthesis of 2,3-BPG from 1,3-bisphosphoglycerate [6], and the fructose-2,6-bisphosphatase (F26BPase) that removes the phosphate moieties of fructose-2,6-bisphosphate [7,8].

Aside from metabolic functions, a surprisingly large proportion of PGM family members are involved in cell signaling and/or regulation. F26BPase [7,8] and TIGAR (tumour protein 53-induced glycolysis and apoptosis regulator) [9–11], two distinct members of the family, have roles demonstrated or strongly suggested respectively, in controlling the concentration of intracellular F26BP (fructose 2,6-bisphosphate) and thereby influencing the rates of glycolysis and gluconeogenesis in eukaryotic cells. Furthermore, Sts-1 (suppressor of T-cell receptor signaling 1) – another PGM family member – has been shown to function as a protein tyrosine phosphatase, dephosphorylating tyrosine kinases (e.g. ZAP-70, Syk, and EGF receptor) [12–16]. This observation expands the regulatory role of PGM family members outside glucose metabolism. More recently, a human member of the family, PGAM5, was implicated as a Ser/Thr protein phosphatase involved in the activation of ASK1 (apoptosis signal-regulating kinase 1) [17].

There are no previous reports characterizing any *Plasmodium* PGMs, although a crystallographic structure for PF11_0208, which we dub *Pf*PGM1, is available in the Protein Data Bank (PDB) [18]. In the present report, we describe the results of a study of the protein encoded by PFD0660w, namely *Pf*PGM2, which shares only 11% sequence identity (23% similarity) with *Pf*PGM1. *Pf*PGM2's enzymatic activity was compared to that of a classical dPGM from a related apicomplexan parasite *Cryptosporidium parvum* with 65% sequence identity (78% similarity) to *Pf*PGM1 and 12% identity (22% similarity) with *Pf*PGM2 (Fig. 1). Furthermore, we determined the three-dimensional structures of *Pf*PGM2 and *C. parvum* dPGM, and superimposed them with other members of the PGM family including *Pf*PGM1. To correlate our structural and biochemical findings on PGM2, we generated a *Plasmodium berghei* strain expressing a GFP (green fluorescent protein) fusion version of the enzyme, which in conjunction with antibodies raised against *Pf*PGM2 allowed us to follow the expression at different parasite stages. Using available transcriptome data for the blood cycle of *Plasmodium*, our results suggest that *Pf*PGM2 is an essential phosphatase at the blood stage where it likely acts upon phosphoproteins.

2. Materials and methods

2.1. Cloning and protein production

The full-length coding region of PFD0660w (PlasmoDB, http://www.plasmodb.org/ [2]) was cloned from *P. falciparum* 3D7 genomic DNA. Full-length *Pf*PGM2 protein and several N-terminal truncated clones were obtained including with an N-terminal 6xHis-tag. Proteins were expressed as previously described [19]. Briefly, clones were grown in TB media in a LEX bioreactor system (Harbinger Biotechnology and Engineering Corp., Ontario, Canada). Overnight starter cultures were left to grow at 37 °C until reaching an OD_{600} value around 5, cooled to 15 °C, and subsequently induced with 0.5 mM IPTG overnight at 15 °C. Cells were harvested by centrifugation and the pellets resuspended in 40 ml per liter of culture in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol, 1 mM benzamidine and 1 mM phenylmethyl sulfonyl fluoride (PMSF), then flash-frozen in liquid nitrogen and stored in −80 °C until needed. Selenomethionine-labeled protein was produced following a modified protocol: M9 SeMet High-Yield growth media (Medicilon) was used instead of TB and the induction OD_{600} was ~1.5. The dPGM construct from *C. parvum* (cgd7_4270) included residues Thr2 to Lys249, it was cloned into a pET28 vector containing an N-terminal 6xHis tag and the expression protocol was the same as described above.

The single-purification protocol was followed for native *Pf*PGM2, *C. parvum* dPGM and selenomethionine-labeled *Pf*PGM2 proteins. The resuspended pellets were pretreated with 0.5% CHAPS and 500 U of benzonase for 40 min at room temperature and cells were mechanically lysed with a microfluidizer (Microfluidizer Processor, M-110EH). The cell lysate was centrifuged to eliminate cell debris and the resulting cleared lysate was loaded onto a DE52 (Whatman, MA, USA) anion exchange resin followed by a 2 ml Ni-NTA (Qiagen, MD, USA). The Ni-NTA column was then washed with 200 ml of a buffer consisting of 50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM imidazole and 5% glycerol. The protein was eluted with 15 ml of a buffer consisting of 50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM imidazole and 5% glycerol. The eluted sample was further purified by size exclusion chromatography on a Superdex 200 26/60 (GE Healthcare, NJ, USA) column equilibrated with a buffer consisting of 10 mM HEPES, pH 7.5 and 500 mM NaCl. The peak fractions eluting at retention volumes consistent with a tetrameric or dimeric enzyme for most clones were collected (Fig. S1D and E), and protein identity was evaluated by SDS-PAGE (Fig. S1B and C) and mass spectroscopy. None of the purified proteins used for enzymatic characterization or X-ray crystallography had its N-terminal purification 6xHis tag removed.

2.2. Enzyme characterization

2.2.1. Phosphoglycerate mutase activity

The cofactor-dependent phosphoglycerate mutase activity was characterized under the following conditions: 50 mM Tris–HCl pH7.5, 150 mM NaCl, 20 mM KCl, 5 mM $MgSO_4$, 1.25 mM ADP, 0.25 mM 2,3-BPG, 18.7 μM NADH, 0.008 U Enolase (Sigma–Aldrich) and 0.05 U of pyruvate kinase and lactate dehydrogenase (Sigma–Aldrich). The concentration of 3-PG ranged from 0.07 to 10.7 mM. The enzyme concentrations used were as follows, 30 ng/μl *C. parvum* dPGM, 50 ng/μl Thr60-Phe295 *Pf*PGM2 and 300 ng/μl Asn100-Phe295 *Pf*PGM2. The reaction was monitored by following the absorbance at 340 nm for 30 min in a Synergy2 plate reader (Biotek). The kinetic parameters were obtained by fitting initial rate against substrate concentration using a nonlinear curve-fitting algorithm (SigmaPlot 2000 software; SPSS Inc., Chicago, USA).

C. parvum enzyme is considered a classical cofactor-dependent PGMase and has been used this study as a positive control for the phosphoglycerate mutase activity.

2.2.2. Phosphatase activity

2.2.2.1. pNPP. A generic phosphatase substrate *para*-nitrophenyl phosphate (*p*NPP) was used to characterize *Pf*PGM2 phosphatase activity. Initially, we determined the optimum pH for hydrolysis using a buffer system composed of citric acid, HEPES and CHES in a pH range of 4–10 [20], 5 mM *p*NPP (Sigma) and 0.1 μg of enzyme. The reaction was incubated for 1 h at room temperature and stopped by the addition of 3 volumes of 1 M NaOH. Subsequent enzyme characterization using *p*NPP as substrate was done under the following conditions: 25 mM Na citrate, pH 6, 250 mM NaCl; *p*NPP concentration was varied between 0.01 and 12 mM. The reaction was incubated for 1 h at room temperature and stopped with