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Trypanosomatid phosphoglycerate mutases have multiple conformational and oligomeric states

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ABSTRACT

Three structurally distinct forms of phosphoglycerate mutase from the trypanosomatid parasite *Leishmania mexicana* were isolated by standard procedures of bacterial expression and purification. Analytical size-exclusion chromatography coupled to a multi-angle scattering detector detected two monomeric forms of differing hydrodynamic radii, as well as a dimeric form. Structural comparisons of holoenzyme and apoenzyme trypanosomatid cofactor-independent phosphoglycerate mutase (iPGAM) X-ray crystal structures show a large conformational change between the open (apoenzyme) and closed (holoenzyme) forms accounting for the different monomer hydrodynamic radii. Until now iPGAM from trypanosomatids was considered to be only monomeric, but results presented here show the appearance of a dimeric form. Taken together, these observations are important for the choice of screening strategies to identify inhibitors of iPGAM for parasite chemotherapy and highlight the need to select the most biologically or functionally relevant form of the purified enzyme.

1. Introduction

The search for chemotherapeutic targets in parasites of the Trypanosomatidae family (comprising the *Trypanosoma* and *Leishmania* genera) has identified the glycolytic enzyme, phosphoglycerate mutase (PGAM) as an attractive prospect [1]. This enzyme occurs in two taxon-specific versions which share no structural or mechanistic similarities. Both versions catalyse the interconversion between 2-phosphoglycerate (2PGA) and 3-phosphoglycerate (3PGA). PGAM in humans belongs to the cofactor-dependent version (dPGAM), whereas the enzyme from trypanosomatids is the alternative cofactor-independent version (iPGAM) [2,3]. Moreover, it is known that the pathogenic bloodstream stage of *Trypanosoma brucei* is completely dependent on the catabolism of glucose to pyruvate via the glycolytic pathway for the production of ATP [4–6]. In addition, RNAi experiments have shown that an approximately 50% reduction in the intracellular level of PGAM in *T. brucei* (*Tb*PGAM) is sufficient to cause death of cultured parasites [7].

Human dPGAM is a dimeric enzyme that occurs in two tissue-specific isoenzymes. Both isoenzymes are dependent on 2,3-bisphosphoglycerate for activity, and involve a phosphohistidine intermediate at the active site. Similar dPGAMs are found in vertebrates, amphibians, yeasts and certain bacteria. By contrast, iPGAMs occur in plants, many protists including kinetoplastids which comprise the Trypanosomatidae, nematodes, algae and certain other bacteria. These are monomeric two-domain enzymes which require divalent metals for activity, and involve a phosphoserine intermediate [8–11].

Analysis of the amino-acid sequences of iPGAMs shows that they may be grouped into two families (Fig. S1 and Table S1). iPGAMs from plants and trypanosomatids comprise family 1, with the remaining enzymes in family 2. X-ray crystal structures are available for *Leishmania mexicana* PGAM [11] and for *Tb*PGAM from family 1 [12], and for *Geobacillus stearothermophilus* [13], *Staphylococcus aureus* (4MY4.pdb) and *Bacillus anthracis* from family 2 [14]. The crystallographic data show that both family 1 and family 2 iPGAMs can be trapped in open and closed monomeric forms. In the present study we investigated which conformational and oligomeric states of *Lmi*PGAMs are present in solution. These findings are of particular relevance for the choice of screening strategies to identify inhibitors of iPGAMs with a view to drug discovery and development.

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2. Materials and methods

2.1. Materials

3PGA, enolase (baker's yeast) and pyruvate kinase (rabbit muscle) were obtained from Sigma–Aldrich; lactate dehydrogenase (LDH, rabbit muscle) from Fluka Analytical. *Escherichia coli* competent cells were from Novagen. Prepacked chromatography columns were obtained from GE Healthcare, and Vivaspin columns from Sartorius Stedim Biotech.

2.2. Expression and purification of LmPGAM

Chemically competent *E. coli* BL21(DE3) cells were transformed with pET28a*Lm*PGAM (C-terminal His-tag; LEHHHHHH)[10]. Cells were grown in 2xTY medium containing kanamycin (50 μg mL^{-1}); 250 rpm shaking and expression induced for 18 h, 291 K, 1 mM IPTG. Cell pellets were re-suspended in 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% glycerol and EDTA-free protease inhibitor (1 tablet/1 L cell culture equivalent; Roche) and lysed with a Constant Systems 1.1 kW TS Cell Disruptor (set to 25 kPsi; 172 MPa). After centrifugation the supernatant was loaded onto a 5 mL IMAC Hitrap HP column, pre-charged with nickel and washed with ten column volumes (CV) of buffer A (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 20 mM imidazole) followed by 15 CV 10% buffer B (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 500 mM imidazole). The *Lm*PGAM protein was eluted with a 10 CV step gradient (10–100% buffer B). Fractions containing *Lm*PGAM were buffer exchanged into 20 mM HEPES, pH 7.6 and 1 mM DTT (Hiprep 26/10 desalting) and loaded onto a Mono Q 5/50 GL ion-exchange column. The protein was eluted over a 15 CV gradient with buffer D (20 mM HEPES, pH 7.6, 1 M NaCl and 1 mM DTT). *Lm*PGAM eluted as three distinct peaks; Iex1 (217 mM NaCl), Iex2 (253 mM NaCl), Iex3 (303 mM NaCl). Samples containing *Lm*PGAM were pooled into three fractions (Iex1, Iex2, Iex3), concentrated (1 mg mL^{-1}) and separately loaded onto a Superdex 200 10/300 GL column pre-equilibrated with 20 mM triethanolamine (TEA–HCl), pH 7.6, 50 mM NaCl. Final purity was greater than 95%.

2.3. Enzyme assay

Continuous coupled iPGAM assays were performed using a Multimode Plate Reader-Molecular Devices M5 instrument, by the addition of 10 μL of iPGAM sample (0.02 mg mL^{-1}) to give a final reaction mixture of 100 μL containing 100 mM TEA–HCl buffer pH 7.6, 1.5 mM 3PGA, 5 mM $MgCl_2$, 50 mM KCl, 0.8 mM NADH, 1 mM ADP, 2 units of enolase, 4 units of pyruvate kinase and 6 units of LDH. The decreased absorbance of NADH over time at A_{340} nm was used to obtain the rate of reaction for specific activity measurements (one unit corresponds to the conversion of 1 μmol of substrate min^{-1} mg^{-1} protein at 298 K) [15].

2.4. SEC-MALS analysis

Size-Exclusion Chromatography coupled to a DAWN HELIOS II™ MALS instrument (Wyatt Technology) and Optilab T-rEX refractometer (Wyatt Technology) was used to determine the molar mass of the proteins in the three ion-exchange fractions. 100 μL of 1 mg mL^{-1} (16 μM) of each *Lm*PGAM ion-exchange fraction was loaded onto on a Superdex 200 10/300 GL column pre-equilibrated with 20 mM TEA–HCl, pH 7.6, 50 mM NaCl, at a flow rate of 0.5 mL min^{-1}, 293 K; elution monitored by absorbance at 280 nm. The on-line measurement of the intensity of the Rayleigh scattering was used to determine the weight average molecular masses (M_w) of the eluted proteins, using the ASTRA™ software (Wyatt Technology). Experiments were repeated in triplicate.

2.5. Structural analyses

Superpositions of iPGAM structures were performed using PyMOL (http://www.pymol.org/) and CCP4 SUPERPOSE [16,17]. The rigid body rotations were calculated by simultaneously superposing the phosphatase domain (residues 3–88 and 332–551) of the *Tb*PGAM apoenzyme (PDB ID 3NVL) and *Lm*PGAM holoenzyme (PDB ID 3IGY) structures (RMS fit of the C-α atoms is 0.5 Å). The resulting coordinates were recorded for both structures. Using the new coordinates, CCP4 SUPERPOSE was used to superpose the transferase domain of the apoenzyme onto the holoenzyme structure (the average RMS fit of the C-α atoms for each chain is 0.3 Å), providing both the centroid and the rotation matrix.

The predicted hydrodynamic properties of the protein molecules were obtained from the program HYDROPRO10 [18]. DYNDOM [19] was used to investigate the molecular movements between the open and closed forms of iPGAM, and the program Surface Triplet Propensities (STP) [20] was used to predict the most likely areas on the iPGAM monomer surface that may interact with another monomer to form a dimer (http://opus.bch.ed.ac.uk/stp/). PISA [21] was also used to study the potential interface between monomers to form dimeric iPGAM.

3. Results and discussion

3.1. Ion-exchange chromatography separates LmPGAM into active forms with different hydrodynamic properties

Bacterially expressed His-tagged *Lm*PGAM has previously been conveniently purified in high yields using a cobalt IMAC column [3,10,22]. The presence of cobalt has been shown to hyper-activate iPGAM, though it is unlikely to be biologically relevant [15]. This has prompted us to redesign the purification protocol. The new protocol for C-terminal $(His)_6$*Lm*PGAM (pI 5.4) uses a nickel (IMAC) column followed by separation on a MonoQ 5/50 GL column into three distinct peaks Iex1, Iex2 and Iex3 (Fig. 1A).

The specific activities of Iex1, Iex2 and Iex3 were 20.0, 19.8 and 15.9 μmol min^{-1} mg^{-1}, respectively. These activities are similar to that of *Tb*PGAM purified through a nickel IMAC column (26 μmol min^{-1} mg^{-1}, [9]). As the activities of the three different forms were quite similar, we were interested in how structural differences could explain the different forms of PGAM. Fractions of each of the three ion-exchange peaks (Iex1, Iex2, Iex3) were pooled separately, concentrated to 1 mg/mL and further analysed by analytical gel filtration. Iex1 and Iex2 eluted as single peaks with retention volumes of 13.89 ± 0.03 and 13.38 ± 0.02 mL, respectively. Under identical conditions Iex3 eluted as two distinct species with retention volumes of 11.62 ± 0.02 (minor peak) and 13.37 ± 0.02 (major peak) mL (Fig. 1B).

Size-exclusion chromatography separates proteins on the basis of their hydrodynamic volume. For similarly shaped particles there are linear relationships between the hydrodynamic radius, R_h, and $\sqrt{\log(K_{av})}$ (where K_{av} is the partition coefficient) and between R_h and $logM_r$. Elution positions of *Lm*PGAM from SEC were compared to calibration curves of well characterised globular protein standards to determine whether Iex1, Iex2 or Iex3 had hydrodynamic properties that equated to a globular monomeric form (Fig. 1B). The calculated R_h values were: Iex1, 32.9 Å (64.1 kDa); Iex2, 35.7 Å (81.0 kDa); Iex3, 46.9 Å (184.1 kDa) and 35.8 Å (81.56 kDa) (Fig. S2).

SDS–PAGE could not distinguish between any of these forms of *Lm*PGAM (Fig. S3) as all ran at the same position close to the calculated mass of 61.8 kDa. Therefore only Iex1 approximates to the